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Investigating The Regulation Of A Fatty Acid Efflux Pump In Methicillin Resistant Staphylococcus Aureus

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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INVESTIGATING THE REGULATION OF A FATTY ACID EFFLUX PUMP IN
METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

by

James Schneider

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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Abstract

Community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) strain USA300 has rapidly achieved pandemic status in the community setting. To persist on human hosts, USA300 requires mechanisms to overcome innate immune defenses of the skin, which include antimicrobial unsaturated free fatty acids (uFFAs). This study evaluated efflux mediated mechanisms of resistance to uFFA. *tet38* encoding an efflux pump that was previously implicated in resistance to palmitoleic acid, was found to have no role in resistance to uFFA. Conversely, the *farE* encoded efflux pump conferred resistance to linoleic and arachidonic acid, but not palmitoleic acid. *farE* expression was induced by uFFA, but not other stresses, and in a fatty acid kinase deficient *fakA* mutant unable to incorporate uFFA into phospholipid, *farE* was constitutively expressed, resulting in increased resistance to uFFA. These findings establish that *farE* is expressed in response to the metabolism of exogenous uFFA in *S. aureus*, and confers an efflux-mediated mechanism of resistance.

Keywords: Staphylococci, anti-microbial lipids, *tet38*, *farE*, *fakA*, efflux pumps

Co-Authorship Statement

The following people contributed to the work undertaken in this thesis:

Heba Alnaseri, who created the original the USA300 *farE::ΦNE* strain, and was involved in the experiments shown in Figure 3.1 A.

Zachariah Scinocca, who worked with me on the creation of the USA300 pGY*farE::lux* strain and performed some preliminary experiments which were later repeated to produce Figure 3.3.

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List of Abbreviations

Amp	Ampicillin
ATc	anhydrotetracycline
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
CA-MRSA	Community Acquired Methicillin Resistant <i>Staphylococcus aureus</i>
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
Erm	Erythromycin
HA-MRSA	Healthcare Acquired Methicillin Resistant <i>Staphylococcus aureus</i>
Km	Kanamycin
LB	Luria Broth
MGE	Mobile Genetic Element
OD ₆₀₀	Optical Density (determined at 600 nm)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
TSB/TSA	Tryptic soy broth / tryptic soy agar
UV	ultraviolet
v/v	Volume/volume (ratio)

List of Units/SI Prefixes

b	nucleotide base
°C	degrees Celsius
CFU	colony forming units
F	farad
k	kilo (10^3)
g	gram
× g	gravitational force
L	litre
M	molar (1 mol/L)
m	milli (10^{-3})
mol	mol
n	nano (10^{-9})
OD	arbitrary unit of absorbance based on OD ₆₀₀
Ω	ohm
PFU	plaque forming unit
μ	micro (10^{-6})
V	volt

1 INTRODUCTION

1.1 Overview of *Staphylococcus aureus*

1.1.1 Description

The Gram-positive bacterium *Staphylococcus aureus* has a long history, well befitting its role as the most pathogenic of the staphylococci. In the 1880s physician Alexander Ogston noted the appearance of what he termed *micrococci* in the pus of patients following surgeries and leading to blood poisoning, sepsis, and death. What Ogston called *micrococci*, or tiny balls, were later renamed *Staphylococcus aureus* in reference to their distinctive gold hue; the name *aureus* arising from the Latin ‘aurum’ (gold) (1-4). The reason for this identifiable golden colour is the carotenoid pigment staphyloxanthin, which, in addition to providing *S. aureus* with its etymology, is an important contributor to *S. aureus*’ remarkable survival abilities, contributing to resistance to oxidative and osmotic stresses (5). *S. aureus* has historically been differentiated from other Staphylococci by its ability to produce the protein coagulase, which causes clotting through conversion of fibrinogen to fibrin (6). Since the 1940s, this trait has been important in a medical setting for identification, although it has since been discovered some *S. aureus* strains are coagulase-negative (6, 7). *S. aureus* is also known for its abilities to resist stress. *S. aureus* is capable of surviving in conditions of up to 3.5 M NaCl and pH conditions as low as 4.9, abilities which serve to make *S. aureus* a difficult to eliminate food pathogen (8, 9). A range of tolerances to various conditions has allowed *S. aureus* to be found in places such as food and small mammals; additionally, *S. aureus* is thought to colonize the anterior nares of almost 30% of humans (10, 11). These tolerances, combined with a large arsenal of virulence factors, have made *S. aureus* capable of causing a wide variety of infections and syndromes, such as carbuncles, scalded skin syndrome, toxic shock syndrome, and infective endocarditis (12).

1.1.2 History of Antibiotic Resistance and USA300

S. aureus, in addition to its impressive abilities to survive stresses such as high salt or acidic conditions, has proven to be remarkably adept at accumulating resistances to medical antibiotics. The discovery of the antibiotic penicillin by Fleming was a watershed event, providing an effective antibiotic for the treatment of soldiers during the Second World War. Penicillin is a member of the β -lactam family; a class of bactericidal antibiotics which inhibit the last step in cell wall peptidoglycan synthesis by binding to the necessary transpeptidase (also referred to as a penicillin binding protein, PBP). By 1942, penicillin resistance was appearing in *S. aureus*, a trend which continued until by the 1960s, over 80% of community and hospital acquired *S. aureus* possessed resistance to this antibiotic (13-15). Penicillin resistance, arising from a penicillinase (a form of β -lactamase) as first identified by Kirby, was originally only present in hospital strains, but it quickly moved into the community by the 60s, a trend remarkably similar to the current struggle with methicillin resistance (14, 16, 17).

In 1959, a semi-synthetic derivative of penicillin was developed with resistance to penicillinases. This drug was known as methicillin and was expected at the time to give physicians a reprieve from the challenges of antibiotic resistance caused by the plasmid encoded of β -lactamase rapidly spread between strains (14, 18). However, in only two years, Methicillin Resistant *S. aureus* (MRSA) appeared through the acquisition of the *mec* cassette, an event which must have occurred prior to the original identification of MRSA in 1961 (19). Notably, the *mec* cassette carries *mecA*, a gene encoding the penicillin-binding protein PBP2a, which has a low affinity to β -lactams and can thus replace the more susceptible PBP enzymes, which are essential for peptidoglycan cross linkage in *S. aureus*'s cell walls (20, 21). Throughout the 70s and 80s, strains of MRSA were mostly restricted to hospitals in large urban centres; however, during the early 90s, there was a large increase of appearances of MRSA in smaller hospitals and even the community (14).

One of the major strains in the rise of community acquired MRSA was the strain USA300. USA300 was distinctive as it possessed a cassette known as the Arginine Catabolic Mobile Element (ACME), thought to have been acquired horizontally from *Staphylococcus epidermidis* (22). As will be discussed later, this cassette is hypothesized to increase the ability of *S. aureus* to colonize the human skin. This ACME cassette is adjacent to USA300's *mec* cassette. Community acquired MRSA strains tend to have shorter *mec* cassettes, which have a lower fitness cost

compared to the longer hospital acquired MRSA *mec* cassettes. USA300 possessed the staphylococcal cassette chromosome *mec*- IV (SCC*mec*-IV), which was only 24 kilobases long (23). Researchers have theorized that the positioning of the *mec* and ACME cassette in USA300 adjacent to one another might cause the methicillin resistance to ‘hitchhike’ along with the fitness enhancing ACME cassette during horizontal gene transfer, thus giving rise to the large dissemination of USA300 in the community (Figure 1.1) (24). By 2004, over 97% of skin and soft tissue infections in North America caused by MRSA were caused by strain USA300 (25). USA300 has thus become an epidemic strain in communities and is responsible for a wide variety of medical issues, which will be subsequently discussed.

1.1.3 Medical Significance of USA300

The increasing prevalence of strain USA300 in the community is of special concern to the medical establishment. In addition to possessing antibiotic resistance, USA300 is more virulent than other CA-MRSA strains, with greater expression of virulence factors, and causing more severe diseases than other epidemiological relevant strains such as USA400 (26). USA300 has been associated with and is one of the largest causative agents in invasive diseases such as infective endocarditis, necrotizing pneumonia and necrotizing fasciitis (27-29). A combination of factors, including a noted ability to survive attack by the immune system, contributes to this ability of USA300 to invade organs in a more aggressive manner than other *S. aureus* strains (30). In addition to causing serious damage to organs, the widespread dissemination of this strain has also contributed to MRSA strains being the leading cause of skin and soft tissue infections in the United States (31). A likely explanation for the spread of this strain is a greater ability to persist on human skin and in nares and thus to colonize and spread. It has been suggested that the ACME element acquired from *S. epidermidis* contributes to the ability of USA300 to survive and persist on the skin through producing enzymes to counter host polyamines found on the skin, while also countering host skin acidity by converting the amino acid L-arginine to carbon dioxide, ATP and ammonia to counteract acidity (32). Additionally, USA300 has the potential to be difficult to treat; in addition to the SCC*mec* providing resistance to methicillin and other β -lactams, USA300 has proven to be adaptable in evolving resistance to some drugs used to treat MRSA, such as vancomycin (33). Overall, USA300 is a bacterium of medical significance due to its antibiotic resistance, its

possession of hyper-virulent qualities during invasion, and its wide dispersal and ability to colonize skin (34).

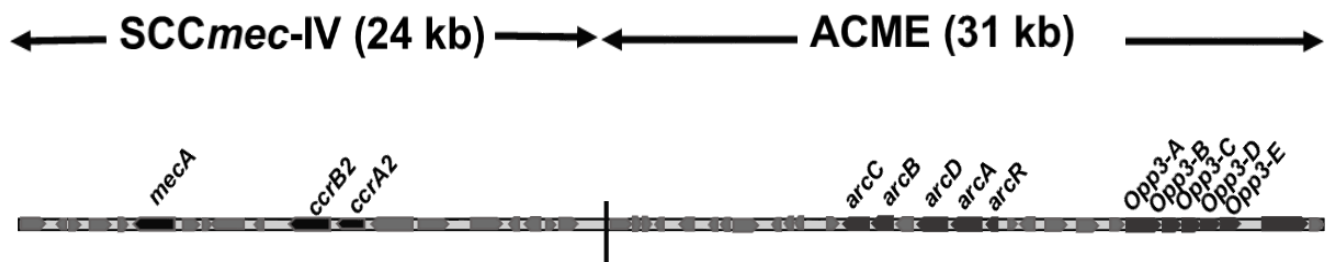


Figure 1.1 Arginine mobile genetic element and SCCmec-IV

The 31 kb ACME cassette is hypothesized to be the result of a horizontal gene transfer from *S. epidermidis* and is thought to provide increased ability to colonize human skin. It is directly adjacent to type IV Staphylococcal chromosomal cassette *mec*, leading some researchers to suggest that *mec* might spread through ‘piggybacking’ with ACME. In SCCmec-IV, *mecA* encodes the PBP2A gene which provides methicillin resistance, while *ccrB2* and *ccrA2* encode cassette chromosome recombinases. In ACME, the *arc* genes together encode an entire complete arginine deiminase pathway which converts L-arginine to carbon dioxide, ATP, and ammonia. The other large gene cluster in ACME are the oligopeptide permease (*opp*) genes, which increase virulence and fitness through a currently not understood mechanism. Figure adapted from Diep *et al.* (22)

1.2 USA300 Colonization and Invasion

1.2.1 USA300 Paradigm of Infection

As discussed earlier, USA300 is a leading cause of soft tissue infections, which can lead to a metastatic infection. To better understand the infection and invasion process of *S. aureus* and thus USA300, the strategies of invasion can be examined in three major stages; colonization, invasion/abscess formation, and metastatic infection.

Initially, there is colonization and attachment of bacteria on the skin, at which point bacteria must survive on the skin in the presence of the host innate immune defenses. Colonization of the skin is a common precursor to the next step, invasion. This has been demonstrated through patients often being colonized by the same strains isolated from the infections (10). The subsequent invasion often occurs from damage to the skin or hair follicles allowing *S. aureus* to breach the defenses of the skin (35). At this point, the bacteria form abscesses, which are formations of pus and bacteria, separated from healthy tissue by a fibrin barrier. It is at this point that *S. aureus* might escape from the abscesses to cause a metastatic infection, the third stage of invasion. Metastatic infection could lead to septicemia in the blood or invasion and abscess formation in other organs (35).

This paradigm of infection also involves the expression of different virulence factors, which are differentially regulated throughout the different stages of infection and will be discussed through this review. Virulence factors are molecules expressed by bacteria which are able to increase their *in vivo* fitness and pathogenicity. In *S. aureus*, they correspond roughly to four major groups: adhesion factors, immune evasion factors, toxins, and tissue degrading enzymes. The regulation of these factors during the different stages of infection is critical to the success of *S. aureus* as pathogen. Interestingly, USA300 is considered to express more of certain virulence factors than other strains of MRSA such as USA400. This is considered by some researchers to contribute to the increased pathogenicity of this strain (26).

1.2.2 Skin Innate Immune Defenses

The first line of defense encountered by pathogens such as *S. aureus* that colonize the skin are the innate defenses of the skin, which, depending on effectiveness, can limit survival and colonization ability. One such defense is the secretion polyamines, notably putrescine, spermidine, and spermine, which are polycationic compounds involved in the regulation of cellular processes such as growth, and are thus elevated in actively growing cells (36). While these amines are extremely important for Eukaryotic cell function and were previously believed to be made by all living things, *S. aureus* does not actually produce them and interestingly they exert bactericidal effects on *S. aureus* at the physiological concentrations encountered on skin (37). Although many bacteria benefit from exposure to polyamines, *S. aureus* is killed in an unclear mechanism involving the compound menaquinone; thus, the detoxification of these amines is important for *S. aureus* survival (37). USA300 is notably capable of growing despite these polyamines as a result of the *speG* found on the ACME cassette, which encodes a detoxifying spermidine acetyltransferase (37).

Another antimicrobial defense on the skin is its acidic pH; skin and sweat naturally have a low pH (median 5.3), which is the result of many factors such as lactic acid, amino acids, ammonia levels, and fatty acids (38). This is well below the optimal pH of many bacteria, including *S. aureus*, which is at approximately 7.0; correspondingly, it has long been noted that patients with a higher natural skin pH are more susceptible to infections (39). USA300 is once again notable as having systems from the ACME cassette which enhance acid tolerance, encoding an arginine deiminase pathway, which counters the acidity of the skin through conversion of L-arginine into carbon dioxide and ammonia (32).

The sebum secreted through sebaceous glands is another important aspect of the skin innate immune defenses; containing lipids in the form ceramides, triglycerides, cholesterol esters and importantly, antimicrobial unsaturated free fatty acids (uFFAs). These antimicrobial free fatty acids contain the majority of the sebum antimicrobial activity, notably the saturated fatty acid lauric acid (12:0) and unsaturated fatty acid sapienic acid (16:1 *cis*- Δ^6) (40). Similarly, fatty acids such as linoleic acid (18:2 *cis*, *cis*- Δ^9 , Δ^{12}) and arachidonic acid (20:4 *cis*, *cis*, *cis*, *cis* Δ^5 , Δ^8 , Δ^{11} , Δ^{14}) are present in nasal secretions as innate antimicrobial defenses for the nares both as free fatty acids and cholesteryl esters (41). The role of uFFAs in prevention of *S. aureus* colonization is evident in studies finding that patients deficient in skin fatty acids are more susceptible to

colonization (42). The importance of uFFAs and their mechanism of action will be discussed in greater detail at a later point in this review.

Recent research continues to identify additional innate immune responses to *S. aureus* within the skin. Notably, adipocytes in the subcutaneous adipose tissue have been demonstrated to have a role in preventing *S. aureus* infection through the release of antimicrobial cathelicidins (43). This finding was of particular interest as adipocytes are not normally considered to be cells involved in the immune system.

Together, the various innate immune defenses discussed here serve to create an environment on the skin which is inhospitable to undesired microbes. For *S. aureus* to be successful in colonization, the first step of the model of invasion, it must be capable of surviving and overcoming innate immune defenses.

1.2.3 Invasion and Formation of Abscesses

When *S. aureus* is able to overcome the barrier of the skin and establish a soft tissue infection, it commonly takes the form of an abscess which is a small build-up of pus. Abscesses in the skin and soft tissues develop when *S. aureus* is able to breach the local skin defenses through cuts or trauma to hair follicles and enter underlying tissues (35). From these initial abscesses, *S. aureus* can either disseminate back onto the skin surface to establish more infection on the skin, or move into the circulating blood to cause a metastatic infection with the formation of abscesses at new sites (44).

When *S. aureus* breaches the innate immune barrier of the skin and accesses underlying tissue, its first step is attachment to host tissue. To accomplish this, *S. aureus* uses adhesion factors associated with the cell wall such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which include the fibronectin binding protein (FnBP)(45). These proteins allow *S. aureus* to bind tissue proteins within the extracellular matrix. This attachment step allow assembly of high numbers of bacteria, which is crucial for the next step of abscess formation.

S. aureus also utilizes virulence factors at this step to resist the innate immune system. One important example is the Staphylococcal protein A (Spa), a cell-wall anchored surface protein that

can bind IgG and make *S. aureus* less susceptible to opsonophagocytosis, which has been linked with more severe disease outcome (46, 47). Other immune evasion factors include the chemotaxis inhibitory protein of *S. aureus* (CHIPS), an excreted protein encoded on a bacteriophage which impairs neutrophil and monocyte response to complement and bacterial formylated peptides, and the multiple peptide resistance factor (MprF), which modifies bacterial membranes to resist binding of antimicrobial defensins (48, 49). The expression of different immune evasion factors is important to prevent the innate system from killing invading *S. aureus* before they can establish large enough numbers.

After *S. aureus* has assembled in high enough numbers within the extracellular matrix, changes in gene regulation cause it to largely change the expression of its virulence factors. Adhesion factors begin to be downregulated and cleaved by proteases, and toxins and tissue degrading enzymes begin to be increased in their expression (35, 50, 51). These toxins serve to both assist in tissue invasion and provide nutrients for *S. aureus*. The destruction they cause gives rise to the pus and begins forming the abscess. The regulation and specific virulence factors expressed at this point will be discussed in further detail in the following section.

As the abscess forms, pro-inflammatory cytokines cause neutrophils to invade the site of *S. aureus* infection, degrading the tissue and causing liquefaction necroses (52). The host also surrounds abscesses with fibrin to attempt to limit the spread of inflammation into healthy tissue, creating a more contained abscess environment (53). *S. aureus* also secretes coagulases to drive the conversion of fibrinogen to fibrin. The overall effect of these responses is while *S. aureus* is contained in the center of the abscess lesion, it is also shielded from host immune cells by the fibrin deposits (54). Within this pseudocapsule is pus, formed from the death of leukocytes and the surrounding tissues and correspondingly made up of their degraded components, including membrane phospholipids. Free fatty acids have also been demonstrated to be found within abscesses (55). These are thought to arise from degradation of host tissue and fatty acid release. Erythrocyte membranes are composed of large amounts of saturated fatty acids, such as palmitic acid (16:0) comprising of about 20% and stearic acid (18:0) at 14%, as well as unsaturated fatty acids, such as oleic acid (18:0 *cis*- Δ^9) at 15%, linoleic acid at 13%, and arachidonic acid at 13% (56). Although these values were determined from phospholipids, the hydrolysis of triglycerides

by leukocyte or staphylococcal lipases within the abscess would cause these fatty acids to also be released as uFFAs (55, 57). Within the abscess *S. aureus* grows in an environment restricted from immune cells and to some extent antibiotics, whereupon it can either disseminate into the blood or continue to enlarge the abscess (58). As USA300 causes a large number of community onset *S. aureus* soft tissue infections, the environment it would encounter within an abscess is extremely important to understand the ability of this strain to invade and cause disease.

1.2.4 Secreted Virulence Factors in Abscesses

S. aureus strains have a wide and varied arsenal of secreted virulence factors with which they can both interact with and kill host cells; in CA-MRSA, large numbers of virulence factors contribute to its virulence and success as a pathogen (59, 60). Virulence factors are molecules produced by pathogens that contribute to their *in vivo* growth and survival through several different mechanisms. Within the abscesses, *S. aureus* reduces its expression of adhesion proteins, and produces large amounts of immune evasion factors, toxins, and tissue degrading enzymes.

One of the most important characteristics that allows USA300 to invade human hosts is an ability to avoid killing by neutrophils, which are first responding phagocytic cells from the innate immune system (60). One proposed explanation for this ability is USA300's acquisition of certain mobile genetic elements (MGE) encoding virulence factors. One common group of MGEs are those encoded on bacteriophage, the Panton-Valentine leucocidin (PVL) is a notable virulence factor encoded on this type of MGE (61). PVL is a two-component toxin which forms pores in leukocytes, causing destruction as well as liberating nutrients; additionally, it causes the release of pro-inflammatory mediators such as IL-8 which drive inflammation (62, 63). Production of PVL has been linked to the increased ability for *S. aureus* strains to form abscesses (62).

In addition to acquisition of mobile genetic elements, USA300 is also known to express its core-genomic encoded virulence genes at higher levels, notably α -hemolysin (Hla; also known as alpha toxin) and the phenol-soluble modulins (PSM) (64). The secreted protein α -hemolysin binds cellular membranes and forms pores that destroy erythrocytes, liberating nutrients in what is termed alpha hemolysis (65). At lower concentrations, α -hemolysin forms a complex with protein ADAM10 (A Disintegrin And Metalloproteinase), which allows it form a pore in the membrane and causes subsequent apoptosis (66). The literature also notes that at higher concentrations, α -

hemolysin non-specifically absorbs into cellular membranes and causes the release of calcium ions and subsequent necrosis (67). The secretion of this factor is an important determinant of virulence in some CA-MRSA models and correspondingly is expressed at greater levels in the USA300 strain than in less pathogenic *S. aureus* strains (68).

Additional core genomic virulence factors expressed at higher levels in USA300 include the phenol soluble modulins PSMs, which contribute to destruction of neutrophils through membrane damage (69). It is likely that the expression of secreted toxins from mobile genetic elements and the increase in expression of core genomic toxin genes both contribute to the remarkable pathogenicity of USA300.

S. aureus strains also secrete enzymes to degrade tissue. One important group of these are the extracellular proteases, secreted enzymes which hydrolyze the peptide bonds that form polypeptides. These secreted proteases have been identified as important contributors to virulence, degrading important host proteins such as cathelicidins (antimicrobial peptides), elastin (an important component of connective tissue) and complement (70-72). In addition to degrading host protein, extracellular proteases also are capable of degrading staphylococcal proteins to effect a change from an adhesive to an invasive state, through acting upon the previously mentioned attachment molecules such as Spa and FnBP (50, 73). As a result, the secretion of proteases is an important step in the formation of abscesses. Several of the *S. aureus* extracellular proteases are expressed as inactive pro-enzymes which can be subsequently activated in a sequence known as the Staphylococcal protease cascade pathway (SPC) (74). This pathway is induced in response to unsaturated free fatty acids, such as those which may be encountered on skin, or in abscesses, thus acting as an environmental signal-response pathway which responds with increased virulence (74). As a result, proteases make up an inducible and varied group of virulence factors in the already large arsenal of USA300. As will be discussed in the next section, the ability of *S. aureus* to sense changes in the environment and correspondingly alter its virulence expression is tightly regulated and extremely important for successful invasion.

1.2.5 Global Regulators and Stress Responses

S. aureus may encounter several different conditions during colonization and invasion, and often needs to induce specific response mechanisms to continue growth and survival in these conditions. In the invasion strategy of *S. aureus*, it is important to downregulate attachment factors and increase production of toxins and tissue degrading enzymes as *S. aureus* moves into abscess formation. Additionally, *S. aureus* needs systems in place to tolerate environment stresses it might encounter. To accomplish this, several systems are in place to regulate genes for virulence and respond to environmental stresses, such as global regulators and sigma factors.

Numerous global regulators, including (but not limited to) accessory gene regulator (*agr*), staphylococcal accessory regulator (*sarA*) and *S. aureus* exoprotein expression (*sae*) systems play important roles in the regulation of virulence factors for *S. aureus* during invasion. Some of these systems, such as *agr* and *sae*, are regulated through two component regulatory system. These systems allow response to environmental signals through autophosphorylation of a sensor histidine kinase in response to stimuli, which begins a cascade terminating with the response regulator binding to specific DNA sequences (75, 76). In the *agr* system, the histidine sensor (AgrC) senses an auto-inducing peptide (AIP), leading to phosphorylation of response regulator (AgrA). This response regulator binds to a specific DNA sequence, encoding the effectors of the system; in the *agr* system, this effector is RNAIII, a small RNA which modulates the expression of numerous virulence genes (76). As AIP is produced by *S. aureus*, and induces the *agr* system in high enough concentrations, it thus acts as a quorum sensing system to sense for sufficient numbers of *S. aureus* (77). It has been observed that this quorum sensing system is important for the establishment of *S. aureus* abscesses; some research has found abscesses are unable to form when the autoinducing peptide is interfered with (78). The regulation of *sarA* is controlled through its growth phase, reaching greatest expression during late exponential phase, which would correspond to establishment in abscesses. Additionally, *sarA* is capable of binding to *agr* promoter regions and activating *agr*. As a result of these systems, *agr* and *sarA* routinely function simultaneously, such as in *S. aureus* establishing abscesses. The *agr* and *sarA* systems are important mediators of virulence, upregulating production of a number of virulence factors such as α -hemolysin, PVL, and toxic shock syndrome toxin (TSST1), while downregulating attachment factors such as Spa (Table 1.1) (51, 76, 79-81). The *sae* system is also involved in expression of secreted virulence factors such as α -hemolysin in a system thought to be independent of *agr* and *sarA* (76). Together,

these systems, and others, allow *S. aureus* to become mobile and establish abscesses with virulence factors. Importantly, *sarA* also has a role in resistance to stressors such as heat or acid (82).

Another important regulator involved in the expression of virulence genes and stress resistance are the sigma factors *S. aureus* uses to direct transcription, notably the alternative sigma factor σ^B (alternatively ζ^B). (83, 84). This factor is both expressed in stationary phase cells and is a part of the response of *S. aureus* to various external stresses such as osmotic stress, acid stress, and oxidative stress (85). Interestingly, studies found that when resistance to either temperature, oxidative or acid stress is induced, *S. aureus* would subsequently possess cross-protection to the other stresses (86). As these stresses are thought to be resisted using different mechanisms, σ^B appears to be involved in a generalized stress resistance (82). There is also a connection between σ^B and global regulator *sarA*; both are involved in the production of staphyloxanthin, a pigment involved in resistance to osmotic and oxidative stresses (5, 87). Correspondingly, σ^B is able to promote expression of virulence factors through increased expression of *sarA*; demonstrating the connection between environmental stressors and expression of virulence factors (84). Relevant to this study, σ^B and *sarA* have also both been identified as upregulated in response to exposure to linoleic and oleic acids, which likely caused the upregulation of several genes involved in general stress responses, notably the class three general stress (CtsR) operon and the genes associated with staphyloxanthin production. These findings suggest that aspects of the *S. aureus* response to fatty acid genes are regulated through these two important and connected systems (88).

While σ^B is the most studied of the alternative *S. aureus* sigma factors, it is notable that recent research has identified another stress response sigma factor, σ^S , which is involved in response to starvation and heat shock but not the other stresses (89). Together with σ^B , these two factors are able to induce protective responses against a wide range of stressors. Overall, it is the integration of these different global regulators and sigma factors that serves to both connect environmental stress to virulence expression and provide a system for global stress regulation. These connections would be beneficial in the colonization and invasion lifestyle of *S. aureus*, where hostile environments such as the skin comprise of multiple stressors, and virulence factor expression is important for abscess formation.

Table 1.1 Regulation of virulence factors by *sarA* and *agr*

Virulence Factor	<i>sarA</i>	<i>agr</i>
Aureolysin (metalloproteinase)	-	+
α -hemolysin	+	+
β -hemolysin	+	+
SspB (cysteine protease)	-	?
SspA (V8 serine protease)	-	+
Toxic Shock Syndrome Toxin I (TSST-I)	+	+
Panton-Valentine leukocidin (PVL)	+	+
Coagulase	+	-
Protein A (SpA)	-	-
Fibronectin-binding protein (FnBP)	+	-

+ upregulated, - downregulated, ? unknown

1.3 Unsaturated Free Fatty Acids

1.3.1 Mechanism of Antimicrobial Fatty acids and Resistance

Antimicrobial free fatty acids are a type of host defense used by a wide variety of hosts, including animals and plants, against an equally wide selection of targets, from bacteria to viruses to fungi (90-92). As discussed earlier, antimicrobial unsaturated free fatty acids such as linoleic acid, palmitoleic acid, and arachidonic acid, are encountered by *S. aureus* both on the skin and in the nasal secretions, and additionally during the formation of abscesses (55, 93). Free fatty acids are chains of carbon atoms capped at one end by carboxyl groups and on the other with a methyl group, making the overall structure amphiphatic. Free fatty acids are routinely produced through the actions of lipases, which cleave fatty acids from their lipid headgroups. Unsaturated free fatty acids, uFFAs, have one or more degrees of unsaturation as a result of double bonds. When these bonds are *cis*, it causes the fatty acid chain to bend and these unsaturated free fatty acids are notably more bactericidal than similar saturated ones (94). Although different mechanisms have been put forward for the bactericidal effects of uFFAs, in *S. aureus*, the main mechanism appears to be membrane disruption and correspondingly the collapse of energy metabolism, which relies on a proton gradient involving the membrane (95). Recent research has expanded this idea, suggesting that the accumulation of unsaturated free fatty acids such as palmitoleic acid (16:1 *cis*- Δ^9), which possess surfactant properties, disrupt the phospholipid bilayer to such an extent that solutes such as ATP, and even larger proteins, are able to diffuse out. This leads to subsequent collapse of all cellular metabolism and cell death (96).

To prevent death, *S. aureus* has several different strategies to resist uFFAs. Through the expression of teichoic acid, a polysaccharide which contributes to the structure of cell walls, these walls are thought to be both better able to prevent fatty acids from entering cells, as well as minimizing damage by slowing the leakage of cellular components through destroyed membranes (96, 97). Another strategy involves the protein iron surface determinant A (IsdA), which functions to render *S. aureus* more hydrophilic and negatively charged, preventing the function of uFFAs, which require hydrophobic interactions (98). One study on *S. aureus* response to uFFA exposure found an increase in the expression of *sarA* and σ^B and increases in the expression of pigment staphyloxanthin, which has been shown to contribute to membrane stability. Additionally, this

study found that *S. aureus* alters regulation of many pathways involved in cellular energetics, possibly a response to the impact on energy production from membrane damage (88). Additionally, some bacteria also possess efflux pumps for which fatty acids are thought to be substrates (99). Use of efflux pumps to remove toxic fatty acids before they reach high concentrations could represent a strategy of uFFA tolerance, which will be examined in this study. As fatty acids are an important part of the skin's innate immune response, the balance between the ability of uFFAs to effect bacterial killing and the ability of *S. aureus* to resist is an important determinant in the ability of *S. aureus* to colonize the skin.

1.3.2 Major Facilitator Superfamily and *tet38*

One mechanism to remove toxic fatty acids from the cytoplasm of a cell involves utilization of an efflux pump, of which multiple different families exist. One family is the Major Facilitator Superfamily (MFS), which is the largest known family of secondary active-transport carriers. This family has a broad range of members, several of which are involved in the efflux of antibiotics and other antimicrobial agents (100). Researchers have identified several efflux pumps from the Major Facilitator Superfamily that are upregulated in abscesses, including a gene encoding a pump called *tet38* (101). The Tet38 protein, as evidenced by its name, was originally identified as providing tetracycline resistance through efflux, and has a 46% similarity to another tetracycline efflux pump, TetK (102). The contributions of *tet38* to fatty acid resistance were analyzed in *S. aureus* strain MW2, an isolate of USA400. Through minimum inhibitory concentration (MIC) experiments, *tet38* deficient mutants demonstrated MICs half that of wild-type MW2 ($\approx 19 \mu\text{M}$ and $38 \mu\text{M}$ respectively) in regards to palmitoleic acid, while having no differences in MIC of linoleic acid. Interestingly, it was also found through over-expression of *tet38*, the MIC of linoleic acid, but not palmitoleic, could be doubled (103). Taken together, these results suggested that the *tet38* encoded efflux pump had specificity towards palmitoleic acid, but when produced in large enough amounts, it would also be capable of transporting similar uFFAs. Similarly, *tet38* was found to be induced by sub-inhibitory levels of palmitoleic acid, and to a lesser extent, linoleic acids (2 fold and 1.5 fold inductions respectively). The researchers suggested these results showed that fatty acids, and not tetracycline, might be the native ligand of this efflux pump. In a subsequent animal colonization model, *tet38* deficient mutants showed five-fold lower survival on mouse skin relative

to wild-type strains, supporting its purported role in toxic fatty acid removal (103). However, this study did not consider the role of *tet38* in the strain USA300, a strain well adapted for colonization of the skin. Prior to this study, this role in USA300 was uncharacterized.

1.3.3 Resistance Nodulation Division Superfamily and *farE*

Another family of transporters that may have valuable roles in fatty acid efflux is the Resistance Nodulation Division (RND) superfamily, a broad family of efflux pumps which catalyze substrate efflux with an H⁺ antiporter mechanism. The RND superfamily is often involved in the efflux of toxic molecules, and members capable of moving fatty acids have been identified in other species, such as AcrAB in *E. coli* (104-106). Additionally, it has been suggested that RND transporters capture their substrates when they are partially inserted in the lipid bilayer, which is where toxic uFFAs congregate (107). Recent research has identified a gene encoding an RND superfamily member which has been implicated in fatty acid efflux, and was subsequently named fatty acid resistance, effector (*farE*)(108). This gene was identified through investigation of a single nucleotide polymorphism (SNP) in a divergently transcribed gene which conferred greater resistance to fatty acids. Analysis of the gene possessing the SNP predicted, with greater than 99% confidence, that it resembles known AcrR family regulators, causing the gene to subsequently be named as a regulator of fatty acid resistance (*farR*)(108). The divergently transcribed effector-regulator pair bears a strong resemblance to *acrB/acrR* paradigm in *E.coli*, in which *acrB* is repressed by AcrR protein through DNA binding to the *arcB* promoter element. In this model, when AcrR interacts with toxic compounds, it stops repressing *acrB*, allowing its expression, thus acting as an environmental sensor (109)(110). This model is further supported by the strong similarity between AcrB and FarE, which was modelled with 80% similarity (108). The role of *farE/farR* as a regulated efflux system for fatty acids was further supported when exposure to palmitoleic acid was found to significantly upregulate *farE* expression (108). Together, this system is the first description of an inducible RND mechanism of fatty acid resistance in a gram positive bacteria. However, the specific inducers of this mechanism and the connection between this mechanism and other efflux pumps, global regulators, and fatty acid metabolism, remained unexplored prior to this study.

1.3.4 Fatty Acid Metabolism

While uFFAs possess anti-microbial effects and are aspects of the innate immune system, it is important to also recognize that uFFAs, like other fatty acids, can be incorporated into *S. aureus* and become involved in *S. aureus* fatty acid metabolism (111).

Fatty acids are one of the fundamental building blocks of life; in bacteria, they are essential for the phospholipids which comprise the cell membranes. All bacteria utilize the bacterial fatty acid synthase II (FASII) system, a multi-enzyme system, to produce fatty acids. (112-115). Fatty acids generated through the FASII process can subsequently be incorporated by different acyltransferases into phosphatidic acid, a precursor to all membrane phospholipids. Notably, *S. aureus* lacks a fatty acid desaturase such as the one encoded by *des* in *Bacillus subtilis*, and thus does not produce unsaturated fatty acids (116). To maintain membrane fluidity, *S. aureus* instead produces branched chain fatty acids, which account for 55-65% of the fatty acids in *S. aureus* membranes (117). *S. aureus* is also capable of taking up and incorporating exogenous fatty acids, which might provide energy saving advantages (115). Some bacteria such as *Escherichia coli* are capable of utilizing fatty acids as a source of energy by breaking them down utilizing β -oxidation. However, according to the annotated genome sequence, *S. aureus* lacks enzymes for fatty acid catabolism and thus it is unlikely that it would use exogenous fatty acids for energy (22, 118, 119). As a result, the metabolic fate of fatty acids produced from exogenous sources is thought to be incorporation into phospholipids.

Until recently, it was not known how exogenous fatty acids were incorporated into *S. aureus*. This changed with the identification of a two-protein enzyme, fatty acid kinase (Fak). This enzyme is composed of FakA, a kinase domain protein, and a fatty acid binding protein, either FakB1 or FakB2, of which the latter was found to demonstrate specificity towards unsaturated fatty acids. After exogenous fatty acids flip across to the inner leaflet of the membrane by the pH gradient, they are bound by FakB and subsequently phosphorylated by FakA (120). The resulting acyl-PO₄ can then either be used by acyltransferases for phospholipid synthesis, or delivered to the FASII cycle for extension (111). It is through this process that exogenous fatty acids, including unsaturated fatty acids, may be incorporated into the membranes of *S. aureus*.

The identification of this fatty acid kinase raises several interesting questions about the nature of this kinase and the role of incorporation of exogenous fatty acids in *S. aureus*. The gene encoding

the fatty acid kinase A, *fakA*, was originally identified in a transposon mutant with altered resistance to the antibiotic dermicidin (121). The gene was named *dak2* due to its predicted similarity to dihydroxyacetone kinase. Interestingly, these mutants demonstrated an altered phospholipid composition, with significantly lower amounts of diphosphatidylglycerol (DPG)(121). Later studies identified *fakA* as the second gene in a two gene operon, where it was subsequently named *vfrB* after the role of this operon in virulence factor regulation. *vfrA*, the first gene in the operon, belonged to a family of alkaline shock proteins, and had a very small effect on expression of α -hemolysin. Interestingly, *fakA* (*vfrB*) was found to be a potent modifier of toxin production; mutants deficient in *fakA* had no expression of α -hemolysin when grown on solid medium, but were found to over-express extracellular proteases, suggesting a *fakA* role in promoting hemolysis and repressing proteases. In an animal model of skin and soft tissue infection, the *fakA* mutant demonstrated a more virulent phenotype, producing abscesses significantly larger in size (122). Intriguingly, the specific mechanism of how *fakA* affects toxin regulation and virulence remains unknown; additionally, it remains unclear whether this regulation is independent of its role in fatty acid metabolism.

1.4 Rationale and Hypothesis

The goal of this research was to elucidate the specific role and regulation of the *farE* mechanism within the USA300 response to uFFAs. Previous studies had identified another fatty efflux pump, *tet38*, although its role had not been characterized in USA300. Additionally, a mutant deficient in the gene *fakA*, an essential gene for exogenous fatty acid incorporation, had been demonstrated in USA300 to be more virulent and produce larger abscesses. These two studies suggest a complex role of uFFAs in metabolism, efflux and virulence. With this preliminary data in mind, we hypothesized that *S. aureus* resistance to unsaturated free fatty acids on the skin and in abscesses is a multifactorial response involving the regulation of fatty acid specific efflux proteins as well as through the incorporation of fatty acids into phospholipids. To test this hypothesis, we pursued three different objectives. The first objective of this study was to evaluate the relative contributions of *tet38* and *farE* in resistance to long chain uFFAs to identify their specificity and importance. To accomplish this, mutants deficient in these two pumps were evaluated for deficiencies in their growth in uFFAs. The second objective was to identify specific inducers and substrate specificity

of FarE through the testing of different fatty acids and other inducers, and evaluating these changes in expression utilizing a promoter-reporter expression system. Finally, the third objective was to evaluate the role of *fakA* in uFFA tolerance and survival and identify how FakA might be involved in the regulation of FarE. To do this, both *farE* expression and tolerance to uFFAs were examined in a strain deficient in *fakA*. Overall, these interconnected objectives allowed us to explore different strategies and factors involved in the *S. aureus* response to uFFAs.

2 MATERIALS AND METHODS

2.1 Storage and Growth of Strains

Bacterial strains used in this study are defined in Table 2.1. Strains were maintained in tryptic soy broth (Difco™ TSB) and 20% glycerol at -80° C. To generate single colonies, strains were streaked from freezer cultures onto tryptic soy agar plates (1.5% Difco™ Agar) with relevant antibiotics when required. Unless otherwise noted, overnight cultures were generated through selection of single colonies which were subsequently inoculated into 3 mL of TSB in 13 mL culture tubes (Sarstedt), and incubated at 37° C with vigorous shaking (200 RPM) for 18 hours. For strains carrying resistance genes, antibiotics were also added at the following concentrations; chloramphenicol (10 µg/mL) and erythromycin (5 µg/mL) for growth of *S. aureus* strains; ampicillin (100 µg/mL) and kanamycin (40 µg/mL) for growth of *E. coli* strains.

Table 2.1 Strains and plasmids used in this study

Strain or plasmid	Description ^a	Source
Strains		
<i>S. aureus</i> :		
USA300	CA-USA300 LAC cured of antibiotic resistant plasmid	(74)
RN4220	Restriction endonuclease deficient lab strain	(123)
NE2336	Transposon insertion in SAUSA300_2489 (<i>farE</i>), Erm ^r	(124)
USA300 <i>farE</i> ::ΦNE	USA300 recipient of transposon insertion from NE2336, Erm ^r	(108)
NE1354	Transposon insertion in SAUSA300_1058 (<i>hla</i>), Erm ^r	(124)
USA300 Δ <i>tet38</i>	USA300 with markerless deletion of <i>tet38</i> (SAUSA300_0139)	This study
USA300 Δ <i>tet38</i> - <i>farE</i> ::ΦNE	USA300 Δ <i>tet38</i> recipient of <i>farE</i> :: ΦNE, Erm ^r	This study
USA300 Δ <i>fakA</i>	USA300 with markerless deletion of <i>fakA</i> (SAUSA300_1119)	This study
USA300 Δ <i>farE</i>	USA300 with markerless deletion of <i>farE</i> (SAUSA300_2489)	This study
USA300 Δ <i>fakA</i> (<i>pfakA</i>)	USA300 Δ <i>fakA</i> complemented with native <i>fakA</i> , cloned in pALC2073, Cm ^r	This study

USA300 $\Delta fakA$ (pfakAREV)	USA300 $\Delta fakA$ with a non-complementing, reversed orientation <i>fakA</i> , cloned in pALC2073, Cm ^r	This study
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E. coli:

DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁻) <i>supE44 thi-1 gyrA relA1</i>	Invitrogen
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Plasmids

pGYlux	<i>E. coli-S. aureus</i> shuttle vector carrying a promoterless <i>luxABCDE</i> operon	(125)
pGY <i>farE</i> ::lux	<i>E. coli-S. aureus</i> shuttle vector carrying a putative <i>farE</i> promoter for <i>luxABCDE</i> operon	This study
pKOR-1	<i>E. coli-S. aureus</i> shuttle vector. contains <i>xyl-tetO</i> , promoter to express antisense <i>secY</i> RNA	(126)
pKOR Δ <i>tet38</i>	pKOR-1 containing upstream and downstream flanking sequences for deletion of <i>tet38</i>	This study
pKOR Δ <i>fakA</i>	pKOR-1 containing upstream and downstream flanking sequences for deletion of <i>fakA</i>	This study
pKOR Δ <i>farE</i>	pKOR-1 containing upstream and downstream flanking sequences for deletion of <i>farE</i>	This study
pALC2073	<i>E. coli-S. aureus</i> shuttle vector with <i>xyl/tetO</i> promoter-operator region	(127)
pAL <i>fakA</i> (+)	1.8 kb promoterless <i>fakA</i> gene segment cloned in <i>KpnI</i> site (+ orientation) for expression from <i>xyl/tetO</i> promoter of pALC2073	This study

pAL <i>fakA</i> (-)	1.8 kb promoterless <i>fakA</i> gene segment cloned in <i>Kpn</i> I site (- orientation); no proper protein expression from <i>xyl/tetO</i> promoter of pALC2073. Acts as negative control.	This study
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^aAbbreviations: Erm^r denotes resistance to Erythromycin, Cm^r denotes resistance to Chloramphenicol

2.2 DNA Methodology

2.2.1 Plasmid Isolation from *E. coli*

All plasmids used in this study are listed in Table 2.1. Plasmid DNA from *E. coli* was prepared using the Presto™ Mini Plasmid Kit (Geneaid) following the manufacturer's instructions. Briefly, 1.5 mL of stationary phase *E. coli* culture were pelleted via centrifugation in a microcentrifuge tube and then resuspended in 200 µL of Solution I /RNase (50mM Tris, pH 8.0, 20 mM EDTA, 100 µg/mL of RNaseA). Cells were then lysed through addition of 200 µL Solution II (200 mM NaOH, 1% (w/v) SDS), and incubated for 2 minutes until lysate was homogenous. The solution was then neutralized with the addition of 300 µL Solution III (guanidine hydrochloride with acetic acid) and inverted several times until a flocculent precipitate formed. Subsequently, the microcentrifuge tube was centrifuged for 8 minutes at 12,300 x g to pellet the insoluble precipitate. The supernatant was then transferred to column and centrifuged for 1 minute. 600 µL of Wash Buffer diluted with absolute ethanol was then added to the column and centrifuged for 1 minute. This step was repeated to remove any protein contamination, and then it was subsequently centrifuged for 3 minutes at 13,000 ×g to dry the column and remove any remaining ethanol contamination. Plasmid DNA was then eluted into a new microcentrifuge tube by addition of 30 µL of warmed (70°C) elution buffer (10 mM Tris-HCl, pH 8.5) to the column and subsequent centrifugation at 13,000 ×g for one minute.

2.2.2 Plasmid Isolation from *S. aureus*

Plasmid DNA isolation from *S. aureus* was accomplished following the same protocol as described for *E. coli* with one modification. Cells resuspended in 200 µL Solution I were supplemented with 50 µg/mL of lysostaphin and incubated at 37°C for 30 minutes to allow lysis prior to addition of Solution II.

2.2.3 Chromosomal DNA Isolation from *S. aureus*

Chromosomal DNA from *S. aureus* was prepared using the GenElute™ Bacterial Genomic DNA Kit (SIGMA) following the manufacturer's instructions. Briefly, 750 µL of stationary phase *S. aureus* cultures were pelleted via centrifugation in a microcentrifuge tube and resuspended in 200 µL of 2.1×10^6 unit/mL solution of Lysozyme supplemented with 50 µg of lysostaphin which was incubated for 30 minutes at 37°C. Cells were then lysed with the addition of 20 µL Proteinase K and 200 µL Lysis Solution C, which was subsequently vortexed and incubated at 50°C for 10 minutes. Simultaneously, a GenElute Miniprep Binding Column was prepared with the addition 500 µL Column Preparation Solution and subsequent centrifugation at 13,000 ×g. The lysate was then prepared for binding by the addition of 200 µL of absolute ethanol, and vortexed for 10 seconds, and was then loaded into the Binding Column. The column was subsequently centrifuged at 5000 ×g. To wash away protein contaminants, the column was then loaded with 500 µL Wash Solution 1, centrifuged at 5000 ×g, and then loaded with Wash Solution Concentrate (containing 70% ethanol) which was centrifuged for 3 minutes at 13,000 ×g to dry the column. Genomic DNA was then eluted into a new microcentrifuge tube by addition of 100 µL of elution solution to the column and subsequent centrifugation a 5000 ×g for one minute.

2.2.4 Restriction Enzyme Digests

Restriction enzymes were purchased from New England Biolabs (NEB). Digestions occurred in 25 µL volumes for 2-4 hours at 37°C. Digested DNA was cleaned using a GenepHlow™ Gel/PCR Kit (Geneaid) according to manufacturer's instructions.

2.2.5 DNA Ligations

DNA ligations were accomplished using a T4 DNA ligase Rapid Ligation Kit (Roche Diagnostics) following the manufacturer's instructions. Briefly, DNA fragments were ligated in 20 µL reaction volumes for 2-4 hours at room temperature, utilizing a 10:1 molar ratio of insert to vector.

2.2.6 *in-vitro* Recombination

DNA fragments possessing *attB1/attB2* sites were recombined into pKOR-1 plasmid utilizing Gateway® BP Clonase II (Life Technologies) following the manufacturer's instructions. Briefly, DNA fragments were recombined in a 10 µL reaction volume containing 15-150 ng *attB* product, 1 µL BP Clonase II, 150 ng plasmid, and TE buffer, for 1 hour at room temperature.

2.2.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for separation and visualization of DNA fragments. Agarose gels (0.8% w/v) were prepared using a 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA) supplemented with 1.5 µg/mL of ethidium bromide to allow visualization. To run gels, DNA samples (typically 5 µL) were mixed with loading buffer and loaded into wells in the gel. Electrophoresis was carried out utilizing a BioRad PowerPac 300 at 110 V for 30-40 minutes. A 1 kb ladder (NEB) was utilized to determine DNA fragment size. DNA fragments were visualized using a Syngene G-Box.

2.2.8 Isolation of DNA Fragments From Agarose Gels

To isolate specific DNA fragments from restriction enzyme digests, fragments were visualized with UV light and excised from agarose gels utilizing razor blades. DNA fragments were then cleaned using a GenepHlow™ Gel/PCR Kit (Geneaid) according to manufacturer's instructions.

2.2.9 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in reactions of either 50 µL (generating DNA for cloning) or 25 µL volume (screening mutants) following protocols outlined by GenScript. Briefly, a 50 µL reaction was composed of 5 µL 10× *Taq* buffer containing Mg²⁺, 1 µL 10 mM dNTP, 1 µL forward primer (100 µM), 1 µL reverse primer (100 µM), 1 µL template (1-100 ng/µL), 41.5 µL sterile Milli-Q water and 0.5 µL *Taq* polymerase (5 units/µL). 25 µL volume reactions maintained the same ratio with all components halved. Oligonucleotides utilized as primers in reactions are listed

in Table 2.2. PCR was carried out utilizing a PTC-100 Programmable Thermal Controller (MJ Research Inc) optimized for specific annealing temperatures and fragment lengths.

2.2.10 DNA Sequencing

DNA sequencing was done at the London Regional Genomics facility of the Robarts Research Institute (London, ON) with samples prepared according to their specifications.

2.2.11 Computer Analyses

Analyses of sequenced DNA and primer design were done utilizing MacVector (MacVector, Inc, Cambridge, United Kingdom). Protein and DNA BLAST searches were performed utilizing the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 2.2 Oligonucleotides used in this study

Primer name	Sequence ^a
Tet38 UP FP ^b	attB1-GAAACGGTTCTATTGCCAG
Tet38 UP RP ^c	ggacctccgaggGTTTAAGTCATCAGCAATGGCTACAG
Tet38 DW FP ^c	ggacctccgaggGTCAGCTTAAATCGTTGGACAC
Tet38 DW RP ^d	attB2-CGCCACCTGATGCTTTTACTTCTAC
GYfarE_F ^e	cccggatccTTGTACGGTGTACGAGTGCG
GYfarE_R ^f	cccgtcgacCGGTGCATTTGTAGCAAGTG
FarE UP FOR ^b	attB1-CAGTTGTTTTAATAGCGATAAGCACG
FarE UP REV ^c	cgacctccgaggCACTATCCATGCAATGACCGC
FarE DW FOR ^c	ggacctccgaggCAAGAAGTGAAACAGCAATCAGCA
FarE DW REV ^d	attB2-TTCTCTACCGTTACGCCACTCCAG
JB13/fakA UP FOR ^b	attB1-GCGTGTGAACGTCTGTTACCAGTCGAAGC
JB8/fakA UP REV ^c	ggacctccgaggCATTTCAAGTTGTCCTCCTAAGCTTTCTTGC
JB3/fakA DW FOR ^c	ggacctccgaggGTTTCATGAAGGTGGACAACCAATTTATC
JB4/fakA DW REV ^d	attB2-GATGACTTTTCTAATCTATTTAGCCATTGC
fakA_COMP-FOR ^h	tttggtaccACAGGCAAGAAAGCTTAGGAGGAC
fakA_COMP-REV ^h	tttggtaccGCAACTCGAGAACGATACTTTTAACC

^aLower case denotes 5'-additions. Restriction sequences are underlined.

^battB1 site GGGGACAAGTTTGTACAAAAAAGCAGGCT for cloning in pKOR-1

^c*SacII*

^dattB2 site GGGGACCACTTTGTACAAGAAAGCTGGGT for cloning in pKOR-1.

^e*BamHI*

^f*SalI*

^g*SacI*

^h*KpnI*

2.3 Transformation and Transduction Methodologies

2.3.1 Preparation of Transformation Competent *E. coli*

CaCl₂ competent *E. coli* DH5 α cells were prepared for transformation following established lab protocol. Calcium chloride promotes plasmid DNA binding to LPS, increasing the capability of cells to transform. Briefly, overnight stationary phase DH5 α cells, prepared as described above, were used to inoculate 400 mL of LB to an OD₆₀₀ of 0.01. When this culture reached mid-exponential phase (OD \approx 0.5), it was placed on ice for 20 minutes to cool cells. The culture was subsequently centrifuged at 4000 \times g to pellet out cells, which were washed through resuspension in 100 mL of 0.1 M CaCl₂, 15% glycerol (v/v). This mixture was subsequently left on ice for 30 minutes before centrifugation again. After centrifugation, the supernatant was discarded and the pellet suspended in 4 mL 0.1 M CaCl₂, 15% glycerol (v/v) for aliquoting into 100 μ L volumes. The competent cells were flash frozen and placed in a -80 °C freezer for storage until use.

2.3.2 Transformation of CaCl₂ Competent *E. coli*

CaCl₂ competent *E. coli* DH5 α cells were transformed with plasmid preparations constructed through previously described DNA techniques. 10 μ L of plasmid preparation were added to an aliquot of thawed competent cells and incubated on ice for half an hour to allow DNA to bind. Subsequently, cells were heat shocked at 42°C for 2 minutes to allow DNA to enter the cells, followed by a 2 minute incubation on ice. Heat shocked cells then received a 900 μ L addition of LB containing relevant antibiotics at a 1/10 dilution of normal to allow resuscitation. These cells were incubated for 1 h to allow recovery before plating on LB agar containing selective antibiotics. Plates were grown overnight and examined for colonies the following day.

2.3.3 Preparation of Transformation Competent *S. aureus*

Electro-competent *S. aureus* (RN4220, USA300 and USA300 derivatives) were prepared for transformation utilizing established lab protocols. Briefly, overnight stationary phase *S. aureus*

cells were used to inoculate 400 mL of TSB to an OD₆₀₀ of 0.01. When this culture reached mid-exponential phase (OD \approx 0.5), it was placed on ice for 10 minutes to cool cells. The culture was subsequently centrifuged at 4000 \times g at 4° C to pellet cells, which were then re-suspended in 40 mL of ice cold 0.5 M sucrose to wash cells. Subsequent incubation on ice, centrifugation, and resuspension steps, in 5 mL and then 4 mL of 0.5 M sucrose, allow cells to be rinsed of any salts. After cells were re-suspended in 4 mL of 0.5 M sucrose they were aliquoted into 100 μ L aliquots, flash frozen, and placed in a -80°C freezer for storage until use.

2.3.4 Transformation of Electro-Competent *S. aureus*

Electro-competent *S. aureus* cells were transformed with plasmid minipreps prepared from other cells. Importantly, RN4220, a restriction endonuclease deficient *S. aureus* strain, could be transformed with plasmid from *E. coli* DH5 α . USA300 and its isogenic variants were transformed with plasmid DNA prepared from RN4220 or USA300 strains. 3 μ L of plasmid preparation were added to an aliquot of thawed competent cells and incubated on ice for half an hour. Subsequently, cells are moved to a cold 2 mm electroporation cuvette (VWR) and electro-porated utilizing a Bio-Rad Gene Pulser II set to 2.5 KV, 200 Ω , and 25 μ F. Electro-porated cells then received 900 μ L TSB containing relevant antibiotics at a 1/10 dilution of normal to allow resuscitation. These cells were incubated for 1 hour to allow recovery before plating on tryptic soy agar containing selective antibiotics. Strains transformed with larger plasmids were plated utilizing top agar (0.8% agar) to provide a slower introduction to antibiotics. Plates were grown overnight and examined for colonies the following day.

2.3.5 Generation of an In-Frame Mutation

Deletion of genes *fakA*, *farE* and *tet38* were generated utilizing the temperature sensitive plasmid pKOR-1 (126) (Fig 2.1). This plasmid possesses several important features to allow in-frame deletion of genes. Briefly, the *cat* gene to allow chloramphenicol resistance for positive selection, *attP* sequences to allow recombination with genetic material possessing *attB* sequences, and the *repF* gene encodes a temperature sensitive protein RepF, which permits replication at 30° C but not at 42°C. It also possesses a *tetR* and *secY570* cassette to allow for negative selection utilizing

anhydrotetracycline, which via *tetR* drives production of antisense *secY*, which is lethal to cells. The use of this plasmid for the deletion of genes is briefly described here utilizing *tet38* as an example. Briefly, for construction of a *tet38* mutant two sequences of approximately 1000 bp flanking the *tet38* gene were amplified with the primers Tet38 UP FP (attB1-GAAACGGTTCTATTGCCAG) and Tet38 UP RP (ggacctccgcggGTTTAAGTCATCAGCAATGGCTACAG) (upstream), and Tet38 DW FP (ggacctccgcggGTCAGCTTAAATCGTTGGACAC) and Tet38 DW RP (attB2-CGCCACCTGATGCTTTTACTTCTAC) (downstream) (Figure 2.1). These products were digested with *Sac*II and ligated to produce a fusion of the upstream and downstream regions flanking *tet38*. This construct was then cloned into pKOR1 through site-specific recombination between the *attP* and *attB*, sites utilizing BP Clonase II (Life Technologies). BP Clonase II was utilized according to manufacturer's instructions; briefly, 150 ng of the *tet38* upstream/downstream ligation and 150 ng of pKOR vector were combined in a 10 µL reaction and incubated at room temperature for 1 hour. The plasmid was subsequently transformed into *E.coli* DH5α, and after verification of the correct structure through restriction enzyme digests and sequencing of the cloned DNA fragment, was then transformed into strain RN4220 as described previously. After selection for Cm^r colonies at 30°C, plasmid was isolated and was then transformed into USA300 through electroporation. To promote integration of the pKOR vector into the target gene via homologous recombination, 3 mL cultures were incubated at 32°C for two hours, after which the temperature was shifted to 42.3°C. After overnight incubation, cells were then plated on TSA + Cm and incubated at 42.3°C to selection for integration of the plasmid with the target gene (Fig 2.2). Single colonies were then selected to grow at 30°C in 3mL cultures with shaking at 180 rpm, allowing pKOR1 to excise from the chromosome. These cultures were then plated on TSA + ATc to select for colonies cured of pKOR1, as the lethal antisense *secY* on pKOR1 was induced by ATc. Colonies were subsequently screened for sensitivity to chloramphenicol, confirming the removal of pKOR1. Deletion of *tet38* was then confirmed through PCR and sequencing (Fig 2.3).

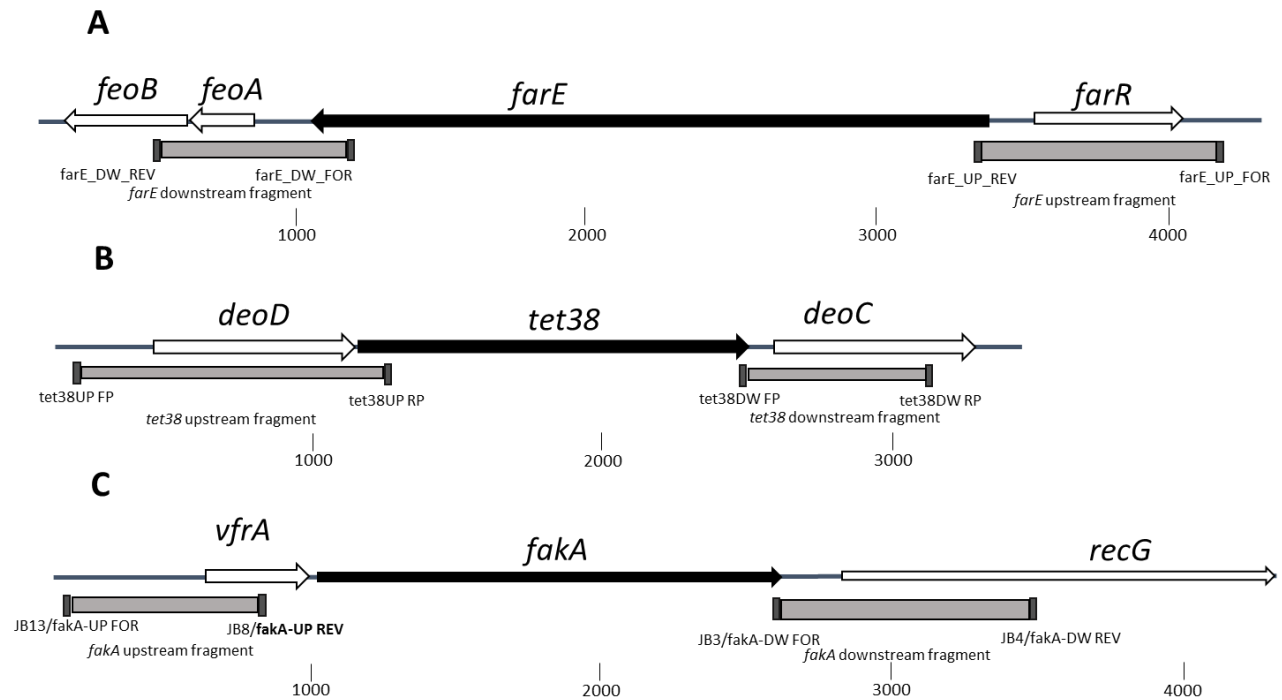


Figure 2.1 Genes deleted with pKOR1 markerless mutagenesis.

Map of *farE* (SAUSA300_2489) (A), *tet38* (SAUSA_0139) (B), and *fakA* (SAUSA300_1119) (C), with primers annotated. Regions between upstream reverse and downstream forward primers will be excised as the upstream and downstream genes are ligated following *Sac*II digestion. *attB* sites are located on upstream forward and downstream reverse primers.

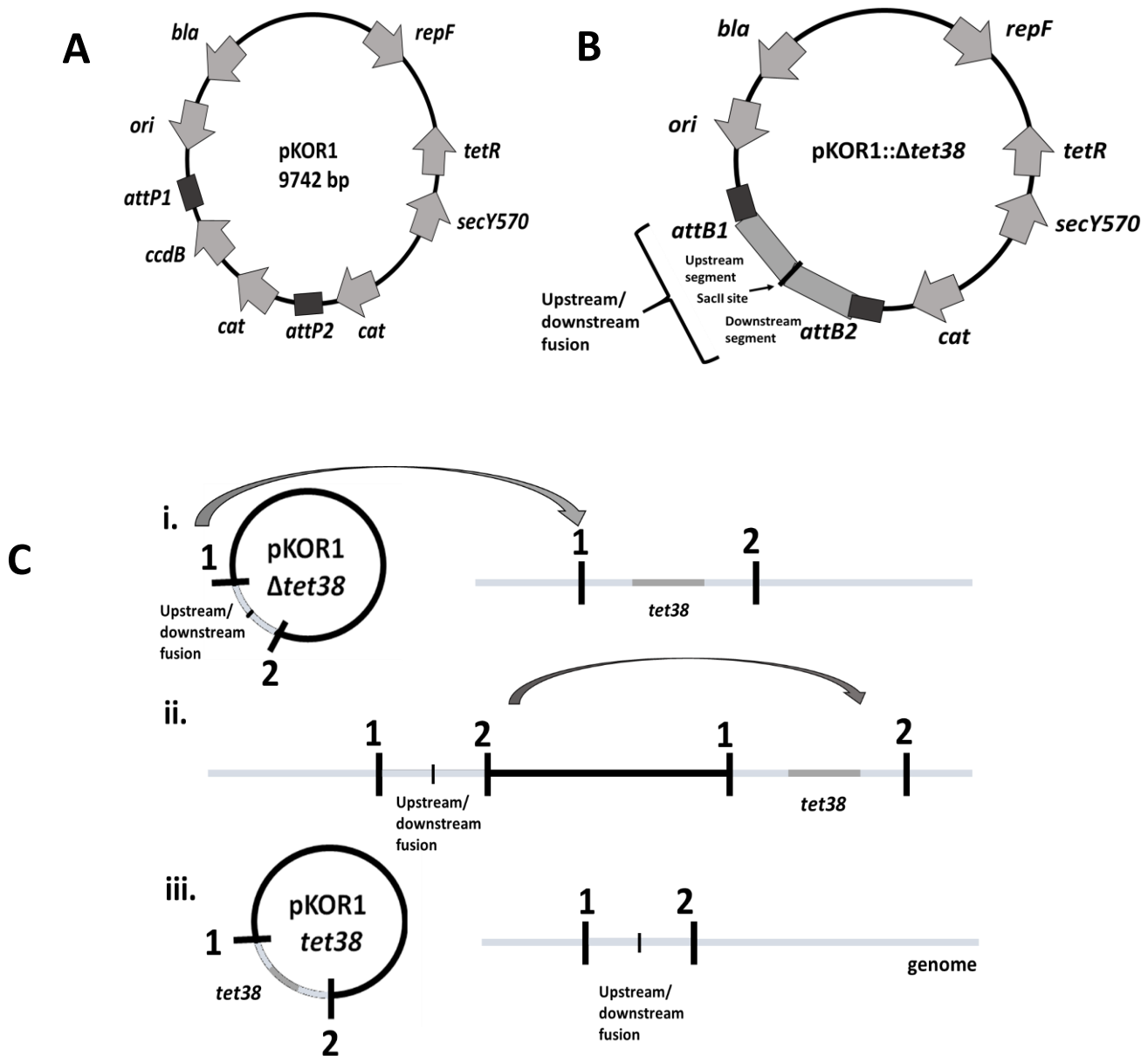


Figure 2.2 pKOR1 structure and recombination.

A. Map of the pKOR1 plasmid. *bla* encodes a β -lactamase to provide ampicillin resistance. *Cat* encodes chloramphenicol resistance. *tetR* and *secY570* together form a cassette in which expresses antisense *secY*, which is lethal to cells when exposed to ATc. *AttP* sequences allow recombination with *AttB* sequences (126). **B.** Map of pKOR1 with $\Delta tet38$ fusion inserted. Not to scale. *attP* sites are lost with recombination. **C.** Hypothetical recombination and resolution with $\Delta tet38$. 'A' and 'B' represent two points of recombination. **i.** Plasmid and genomic DNA both present in cell. **ii.** Following heat shift, pKOR1 recombines into genome at '1'. **iii.** Plasmid is excised along with genomic *tet38* through '2', leaving $\Delta tet38$ fusion in the genome. Further selection eliminates pKOR1 plasmid.

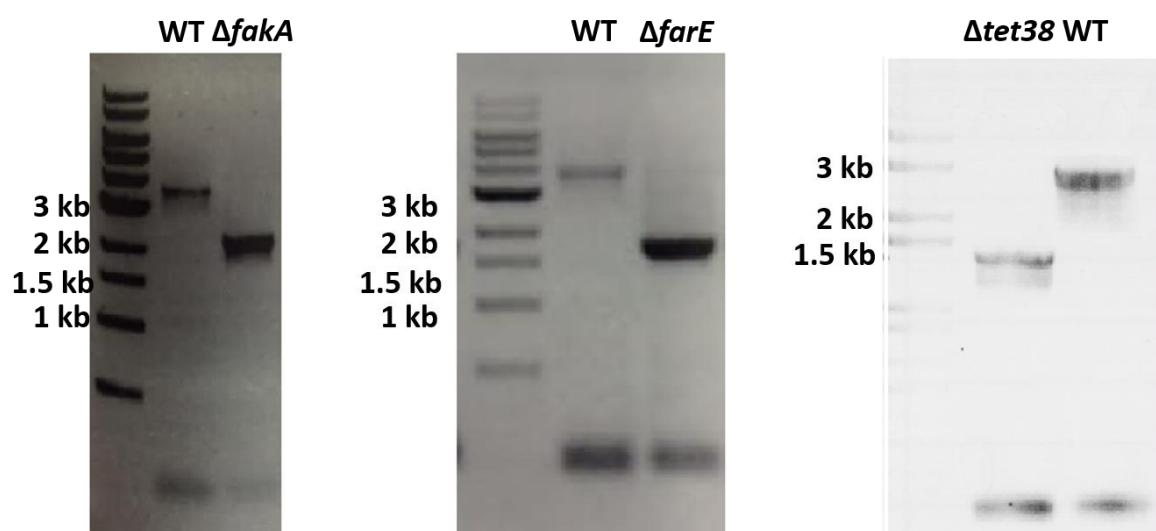


Figure 2.3 PCR Confirmation of Gene Deletions

Wild-type and deletion mutant genomic DNA was used as a template for PCR with primers flanking the deleted genes. Difference in sizes correspond to deletions. Genes; *fakA* (A), *farE* (B), and *tet38* (C).

2.3.6 Phage Transduction

Phage transduction was utilized to generate mutants deficient in target genes by inserting large genetic elements into the middle of genes and correspondingly disrupting gene expression. Transduction is accomplished through shuttling the *bursa aurealis* transposon from the USA300 JE2 Nebraska Transposon Mutant Library into recipient laboratory strains (124). Transducing phage lysate from NTML strains were produced using the phage $\Phi 80$. Donor cells were grown to exponential phase at 37°C with shaking ($OD_{600} \approx 1.0$) and then mixed with dilutions of phage $\Phi 80$ in phage buffer (1 mM $MgSO_4$, 4 mM $CaCl_2$, 40 mM Tris, pH 7.8, 0.1 M NaCl, 1 g/L gelatin). After incubation for 5 minutes at room temperature, these mixtures were plated with top agar on TSA plates containing 4 mM $CaCl_2$. Phage was recovered by gently rocking the plates with 5 mL phage buffer, and then disrupting the top agar with a scraper before centrifuging out cells and agar and filtering the mixture through a 0.45 μm membrane filter. Phage titre of the transducing lysate was then determined using USA300 as an indicator. For transduction, recipient strains were grown overnight in TSB-C (TSB containing 0.5 mM $CaCl_2$). Subsequently, strains were sub-cultured into 50 mL of TSB-C, and then grown to exponential phase at 37°C with shaking. After determination of OD_{600} , the cells were centrifuged and the pellet was re-suspended in TSB-C to achieve a cell density of 5×10^{10} cfu/mL. Subsequently 0.6 mL aliquots were centrifuged and re-suspended in 0.6 mL of transducing phage at 5×10^9 pfu/mL, to achieve a multiplicity of infection of 0.1. After a 10 minute incubation at room temperature, 1.5 mL of TSB-C was added. After a 20 minute incubation at 37°C, 1.0 mL of 2 mM sodium citrate was added to chelate calcium, and the cells were harvested by centrifugation, re-suspended in 1 mL TSB-C, and then plated on tryptic soy agar containing 2.0 mM sodium citrate and 10 $\mu g/mL$ erythromycin. The plates were incubated overnight and evaluated for growth. Single colonies were then selected and transposons confirmed through PCR utilizing primers on flanking regions.

2.3.7 Construction of a pGY*farE*::*lux* Reporter Strain

To construct a pGY*farE*::*lux* reporter strain where the *farE* promoter directly promotes expression of the luciferase operon, a 396-bp fragment containing the promoter site (the intergenic region between *farE* and *farR*) was amplified with the primers GYfarE_F and GYfarE_R. After PCR clean-up and digestion with endonucleases *Bam*HI and *Sal*HI (described above), this segment was

ligated into pGY*lux* (Fig. 2.4) which had previously been digested with *Bam*HI and *Sal*HI. This plasmid was then transformed into DH5 α , RN4220, USA300, and USA300 derivatives, in this order (described above).

2.3.8 Generation of a Double Knockout Mutation

To generate mutants which were deficient in two separate genes, the one gene was deleted with markerless mutagenesis utilizing pKOR, while the subsequent gene was inactivated utilizing phage transduction of a transposon (both described above). This methodology was utilized to produce USA300 Δ *tet38-farE*:: Φ NE as well as USA300 Δ *fakA-farE*:: Φ NE.

2.3.9 Construction of a Complementation Vector

To restore a deleted gene to a strain and demonstrate that an observed phenotype is complementable, the vector pALC2073 was used to restore *fakA*. Briefly, PCR utilizing primers *fakA*_COMP-FOR and *fakA*_COMP-REV were used to generate the *fakA* gene. This product was then digested with *Kpn*I and ligated into the pALC2073 shuttle vector. After transformation into *E. coli* DH5 α , transformants were screened through restriction enzyme digest and sequencing to determine the orientation of the *fakA* insert with respect to the *xyl/tetO* promoter. Plasmid pAL*fakA*(+) contained the 1.8 kb insert cloned into the + orientation for proper expression from the *xyl/tetO* promoter, while pAL*fakA*(-) contained the insert with the opposite (-) orientation, and was used as a negative control.

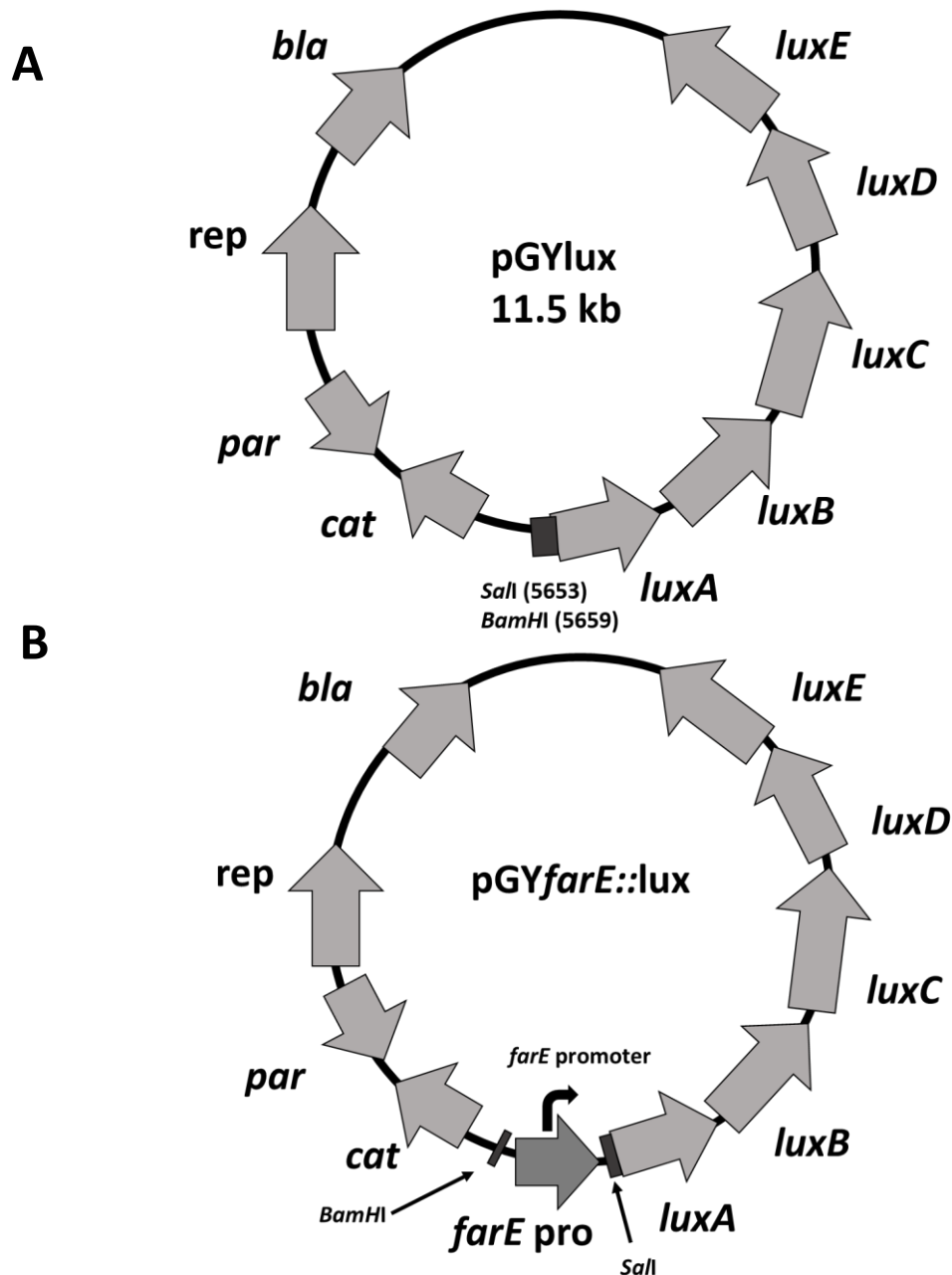


Figure 2.4 pGYlux reporter design

A. Map of the pGYlux plasmid. *bla* encodes a β -lactamase to provide ampicillin resistance. *luxAB* encode bacterial luciferase, while *luxCDE* encode proteins which protein a fatty acid aldehyde substrate. *cat* encodes chloramphenicol resistance. Promoters are cloned between the *Sall* and *BamHI* (125). **B.** Map of pGY*farE::lux*. Intergenic region between *farE* (SAUSA300_2489) and *farR* (SAUSA300_2490) suspected to contain *farE* promoter cloned to drive expression of luciferase genes with *farE* promoter activity

2.4 Experimental Methodologies

2.4.1 Growth Analysis

To evaluate the effect of antimicrobial fatty acids on growth of *S. aureus* USA300 or its isogenic variants, overnight cultures were inoculated into 25 mL volumes of TSB ($OD_{600}=0.01$) supplemented with the indicated concentrations of fatty acids. Briefly, concentrations of 20 μ M linoleic acid (25 μ M palmitoleic) were utilized for what this paper describes as sub-inhibitory concentrations, which are concentrations which slightly retard growth. 100 μ M linoleic was utilized for bactericidal concentrations, which, while not entirely inhibitory, are effective to kill large amounts of the population. Optical density of overnight cultures, prepared as described above, were determined using a spectrophotometer set at 600 nm (OD_{600}). Unless otherwise indicated, all growth assays were conducted in 125 mL Pyrex Erlenmeyer flasks containing 25 mL of TSB and incubated in a 37°C incubator with orbital shaking at 180 RPM.. For supplementation, a stock solution of 5 mM was produced by diluting pure fatty acids in 5 mL TSB with 0.1% DMSO (v/v) and vigorous vortexing. These stocks were then utilized to supplement the cultures to appropriate concentrations. Growth cultures also were supplemented with 0.1% DMSO (v/v) to ensure fatty acid dissolution. Measurements of OD_{600} were taken hourly utilizing a Varian 50 Bio spectrophotometer.

2.4.2 Bactericidal Assays

Overnight pre-cultures grown under conditions described above were inoculated in 25 mL TSB cultures with or without fatty acids as previously explained. These cultures were then grown to mid-exponential phase (2-3 hours; $OD \approx 0.5$ under conditions outlined in Growth Analysis). These cultures were then inoculated into 25 mL TSB flask cultures with bactericidal (100 μ M) concentrations of uFFAs and 0.1% (v/v) DMSO and grown with conditions described previously. Cultures were prepared as quadruplicate. To determine bactericidal activity, aliquots were withdrawn hourly, diluted and plated at 10^0 - 10^{-3} on TSA with quadruplicate technical replicates. After growth overnight colonies were then counted and viable CFU/mL counts determined.

2.4.3 Luciferase Assays

Luciferase assays were conducted under growth assay conditions described above. Cultures possessing the pGY*farE::lux* plasmid were prepared in either triplicate or quadruplicate as specified in figure legends. At specified time points, aliquots were removed from each culture for determination of OD₆₀₀ while concurrently 4 × 200 µL technical replicates were withdrawn from each flask for quantification of luciferase activity. Specifically, each 200 µL aliquot was added to individual wells of an opaque white 96 well micro titre plate (Greiner Bio-One). The wells were then supplemented with 20 µL of 0.1% (v/v) decanal in 40% ethanol, followed by immediate measurement of luminescence utilizing a Biotek Synergy H4 Hybrid Reader, with 1 second of integration and a gain of 200. Background was removed from the relative light units through averaging the technical replicates and subtracting the observed relative light units from a promoterless pGY*lux* reporter. This data was then standardized by dividing with optical density to produce RLU/OD measurements.

2.4.4 Evaluation of non-uFFA inducers

To determine analyze the level of induction of the pGY*farE::lux* promoter reporter in non-uFFA conditions, conditions had to be developed which would be comparable to uFFA exposure. Conditions for these stressors were empirically determined through growth of triplicate USA300 pGY*farE::lux* cultures in 20 µM linoleic acid and in three variations of the stressor conditions, approximated based upon previous observed literature. The conditions which retarded growth, determined as described above, to levels comparable to 20 µM linoleic acid were selected for use in luciferase assays. The assays were subsequently repeated under the determined optimal conditions and levels of expression measured as explained previously.

Several different non-uFFA stressors were selected based upon the literature which are briefly outlined here. Elevated concentrations of sodium chloride were used to examine osmotic stress, which occurs when osmotic environments are abruptly altered (85). Ethanol was utilized to evaluate the effects of alcohols, which possess antimicrobial properties likely through membrane damage and protein denaturation (130). Hydrogen peroxide was utilized to test oxidative stress, which involves free radicals damaging cellular components (85). Hydrochloric acid buffered with MES was utilized to test acid stress, which involves a low pH which can damage cellular components (85). Culture tubes with limited headspace were utilized to test oxygen limitation,

which limits oxygen as an external electron acceptors for *S. aureus* energy production (131). Cadmium chloride was utilized to test toxic heavy metals, which damage membranes, DNA structure, and enzyme functions (132). Deoxycholic acid was utilized to test bile acids, which act as detergents to damage membranes (133). Tetracycline was utilized to test the effects of protein synthesis inhibitor antibiotics (134). Daptomycin was utilized to test membrane disrupting antibiotics, which disrupts membrane fluidity and charge (135). Finally, the oil of *Melaleuca alternifolia* (tea tree) was tested, which acts to disrupt membranes (136). Together, these various conditions provided a broad selection of challenges, many of which have already characterized stress responses.

2.4.5 SDS-PAGE

To analyze the secreted proteins, supernatants were assessed utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Secreted proteins were acquired from the supernatants of 25 mL cultures shaking at 180 rpm at 37°C for 24 hours. The cultures were centrifuged at 3000 ×g for 20 minutes, and then the supernatants are removed for use. A volume of supernatant equivalent to 3.0 OD₆₀₀ units was extracted and incubated on ice for 1 hour with an equal volume of trichloroacetic acid (TCA) to precipitate supernatant proteins. These pellets were then spun at 13,000 ×g for 15 minutes. They were then washed twice utilizing 70% ethanol and dried before being resuspended in 25 µL of 1 × Laemmli buffer. The solutions were then boiled for 5 minutes at 100°C before being centrifuged for 1 minute to collect any evaporated buffer. The entire samples were then loaded into 8% bis-acrylamide gels and run at 120 volts for approximately 90 minutes. The gel was then stained with Coomassie blue for 18 hours (128). Gels were destained with a buffer composed of 40% methanol, 10% acetic acid and 50% dH₂O (by volume) and visualized.

2.4.6 Hemolysis Assay

For hemolysis assays, overnight cultures were diluted in TSB to achieve an optical density of 1.0 (OD₆₀₀). 1 µL of this suspension was then pipetted onto a tryptic soy agar plate containing 5% defibrinated rabbit blood. These plates were subsequently incubated for 37°C for 24 h and then imaged.

2.4.7 Statistical Analysis

All data generated by the assays described below were plotted using Graphpad PRISM software, version 6.0f. Significance at specific time points was determined using unpaired one-tailed Student's *t*-tests, and ANOVA tests followed by Tukey's Range Tests, utilizing the statistics package of Graphpad PRISM.

3 RESULTS

3.1 Relative Contributions of *farE* and *tet38* Efflux mechanisms

3.1.1 Importance of *farE* for Resistance to Linoleic Acid

One of the objectives of this project was to determine the roles of the efflux pumps encoded by genes *farE* and *tet38* (108). Previous research involving *tet38* had determined that *tet38* had an important role in the efflux of palmitoleic acid (16:1 *cis*- Δ^9), while the wild-type had an MIC of 38 μ M PA, it was only 19 μ M PA in *tet38* deficient strains (103). Based on previous research regarding *farE* being important for survival in linoleic acid, it was hypothesized that the two pumps might have parallel functions, with *farE* and *tet38* playing roles in the efflux of linoleic and palmitoleic acid respectively (108). Growth experiments with USA300 and USA300 *farE*:: Φ NE confirm the importance of *farE* for growth in sub-inhibitory concentrations of 25 μ M LA. While USA300 is also slowed compared to growth in TSB alone, USA300 *farE*:: Φ NE has a lengthy 10 hour lag phase before it begins growing when inoculated into TSB containing 25 μ M LA (Figure 3.1 A). This difference in growth in sub-inhibitory LA was not seen in a USA300 Δ *tet38*, consistent with the literature suggesting no changes to the MIC (Figure 3.1 B).

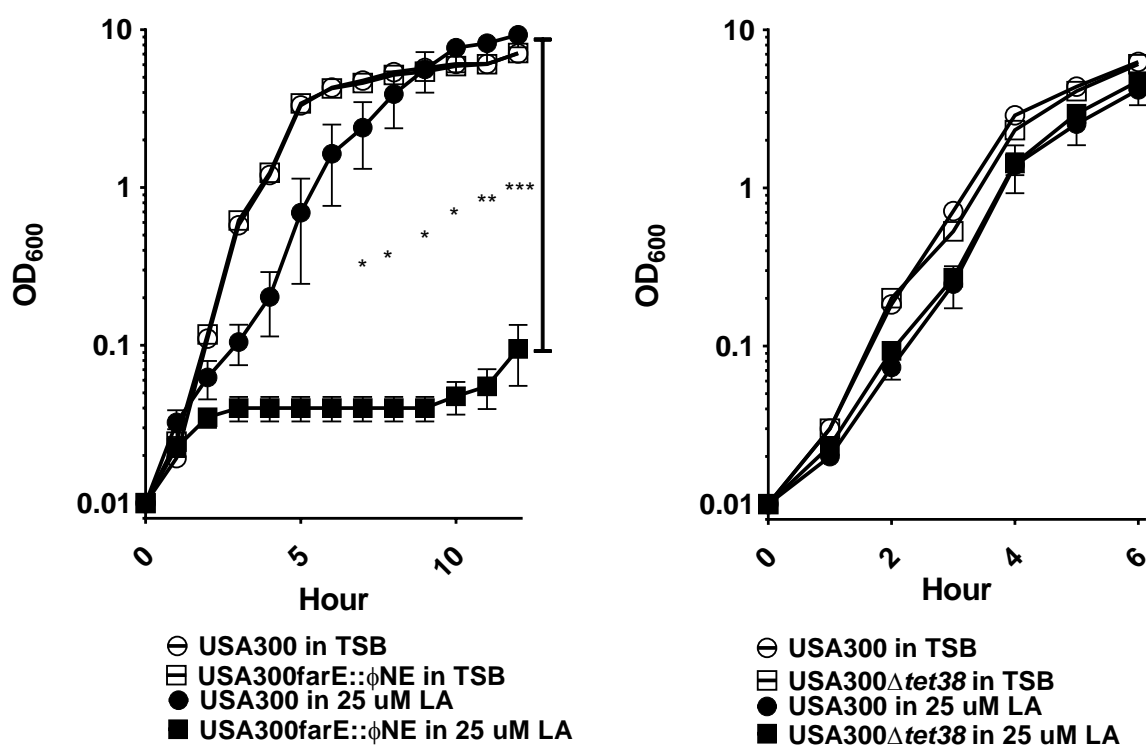


Figure 3.1 *farE*, but not *tet38*, is involved in USA300 growth in linoleic acid

Growth analysis of USA300 and USA300 Δ *farE*:: Φ NE or USA300 Δ *tet38* cultured in TSB or in TSB–25 μ M linoleic acid. Each data point represents the mean value of quadruplicate cultures. Error bars represented by the Standard Error of the Mean. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

3.1.2 *tet38* is Not Important for Palmitoleic Acid Resistance in USA300

As previous research had described the role of *tet38* in palmitoleic acid efflux in the MW2 (USA400) strain of *S. aureus*, we attempted to replicate this finding in USA300. To evaluate the role of *tet38* in USA300 growth in palmitoleic acid, we constructed a *tet38* in-frame deletion as described in the Materials and Methods section. In an attempt to elucidate this phenotype, USA300, USA300 Δ *tet38* and USA300 *farE*:: Φ NE were grown in a sub-inhibitory concentration of palmitoleic acid (25 μ M). While our interpretation of this data reinforced the hypothesis that *farE* does not have a strong role in survival in palmitoleic acid, unexpectedly, there was no difference in growth between the USA300 and USA300 Δ *tet38* strains (Figure 3.2). Moreover, when palmitoleic acid concentrations were increased to a point where they began to slow wild-type USA300 growth (40 μ M), there remained no detectable difference between the growth of USA300 and USA300 Δ *tet38*. To evaluate whether this absence of phenotype was the result of *farE* and *tet38* compensating for one another, a strain deficient in *farE* and *tet38* was constructed by transducing the *farE*:: Φ NE mutation into USA300 Δ *tet38*. This strain USA300 Δ *tet38*-*farE*:: Φ NE was evaluated utilizing the same conditions the single mutants were evaluated with (Figure 3.2). Once again, no difference in growth was detected in palmitoleic acid, indicating that *farE* does not compensate for the deletion of *tet38*. The absence of any growth phenotype for USA300 Δ *tet38* in palmitoleic acid at these different concentrations indicated that *tet38* is not required for growth in the presence of sub-inhibitory concentrations of palmitoleic acid in USA300.

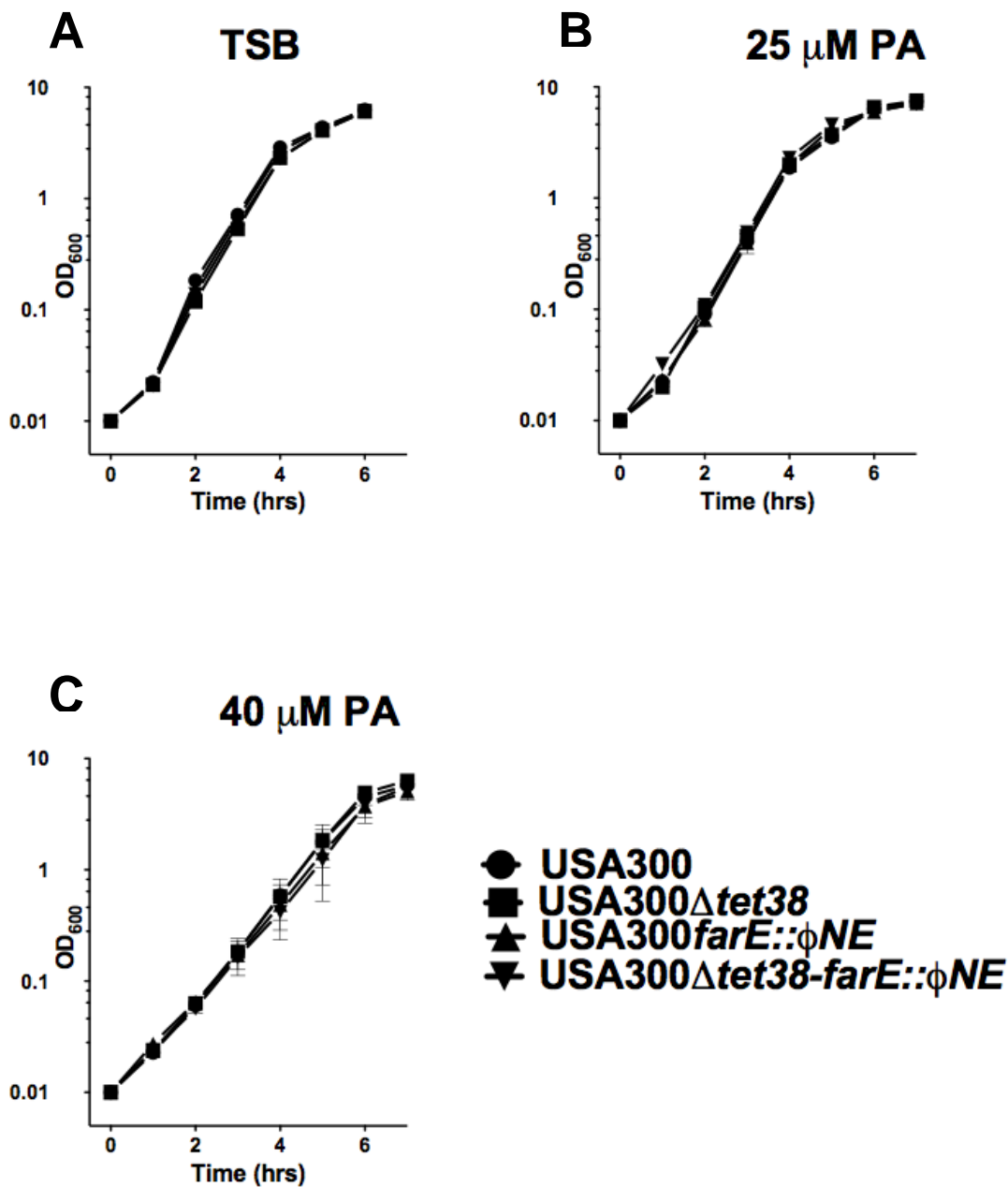


Figure 3.2 *tet38* not required for growth of USA300 in palmitoleic acid

Growth analysis of USA300, USA300 Δ *tet38*, USA300*farE*:: Φ NE and USA300 Δ *tet38*-*farE*:: Φ NE, cultured in TSB, TSB-25 μ M palmitoleic acid or in TSB-40 μ M palmitoleic acid. Each data point represents the mean value of triplicate cultures.

3.2 Inducers of *farE* expression

3.2.1 *farE* is Upregulated in Response to Exposure to Linoleic Acid

It has been demonstrated that *farE* is not only important for resistance to the uFFA linoleic acid, but that a resistance to uFFAs can be induced through growth in sub-inhibitory concentrations (108). In respect to this, we became interested in the manner by which *farE* expression is regulated in USA300 growing both in TSB alone and in the presence of uFFAs. To evaluate the level of expression, the putative promoter of *farE* was fused to the luciferase operon *luxABCDE* on the plasmid pGY*lux* and transformed into USA300. Through monitoring luciferase activity at different time points in growth, we were able to evaluate the expression of *farE* at these different times in growth. Interestingly, we found the level of *farE* expression in USA300 growing in TSB alone to be low but still present, supporting previous transcriptome data (Figure 3.3)(108). This expression was also noted to be highest while USA300 was at an OD₆₀₀ between 0.1 and approximately 0.5, corresponding roughly to the mid-logarithmic growth phase. As anticipated, the expression of *farE* in response to a sub-inhibitory concentration of linoleic acid was found to be significantly higher at every time point measured with the exception of the first. This is likely due to the large amount of background relative to the small number of cells at this point. Similar to the un-induced condition, USA300 in sub-inhibitory LA expressed *farE* at highest levels during the mid-logarithmic growth phase, although it did continue expressing *farE* at significant levels even as it entered stationary phase. In this experiment, USA300 in the presence of 20 μ M LA was demonstrated again to be capable of growth, although at a slightly slower rate than when USA300 was grown in TSB alone. This experiment confirmed the role of sub-inhibitory concentrations of linoleic acid as an inducer of *farE*.

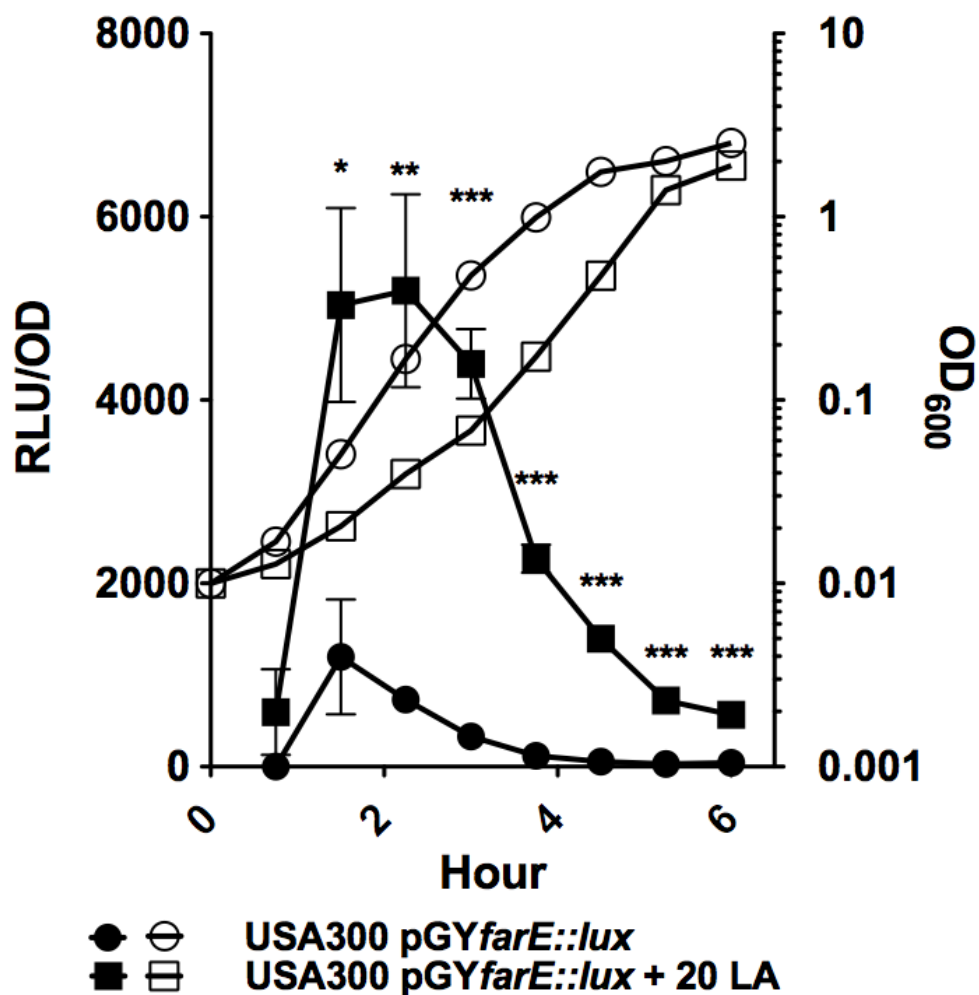


Figure 3.3 Linoleic acid induces *farE* expression.

Growth (OD₆₀₀; open symbols) and relative luminescence units normalized by OD (RLU/OD; closed symbols) of USA300 carrying the pGY*farE*::*lux* reporter vector are graphed. USA300 was grown in TSB alone or in TSB supplemented with 20 μ M linoleic acid. Each value represents the mean and standard deviation of results of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represented by the Standard Error of the Mean. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ determined by one tailed Student's t-test.

3.2.2 *farE* is Induced by Several uFFAs, Notably Arachidonic Acid

Upon confirming the induction of *farE* expression by antimicrobial linoleic acid, as well as identifying the point in growth where optimal expression occurs, we evaluated the specificity of *farE* induction in relation to other uFFAs which *S. aureus* would encounter on skin and in abscesses. As discussed earlier, *S. aureus* is likely to encounter uFFAs both during colonization of the skin or nares and during invasion and abscess formation. As abscesses have been shown to contain large amounts uFFAs due to the action of lipases and the degradation of membrane phospholipids and triglycerides, we wanted to evaluate levels of induction from fatty acids present in the phospholipid membranes. Phospholipid membranes contained large amounts of stearic, oleic, linoleic and arachidonic acids, and to a lesser extent linolenic acid (55, 57). The saturated fatty acid lauric acid and unsaturated fatty acid sapienic acid are both thought to be present in large concentrations in the sebum while linoleic acid and arachidonic acid are located within nasal secretions (40)(41). Small amounts of the uFFA linolenic acid are also present on skin (129).

To evaluate the specificity of *farE* induction, USA300 + pGY*farE*::*lux* was grown to an OD₆₀₀ of ≈ 0.5 in either TSB or TSB supplemented with 20 μ M fatty acid and assayed for luciferase activity (Figure 3.4). There was a large difference in levels of induction within the 18 carbon fatty acids, such that neither stearic acid nor oleic acid caused any significant induction, while linoleic acid and linolenic acid induced significantly greater amounts of *farE* expression. Notably, linoleic acid was able to induce significantly higher *farE* expression than linolenic acid. Additionally, 16 carbon fatty acids palmitoleic acid and sapienic acid were found capable of inducing *farE* expression, although at significantly lower levels than linoleic acid. The 12 carbon medium chain saturated lauric acid, despite having an antimicrobial effect, did not induce any *farE* expression. Strikingly, the 20 carbon uFFA arachidonic acid was the strongest inducer of any fatty acid tested, having significantly greater expression over the next closest, linoleic acid. These findings suggest that linoleic acid is one of the main inducers of *farE* expression while also identifying arachidonic acid, as another important inducer and possible substrate. Additionally, these results demonstrate that palmitoleic acid and sapienic acid are inducers of *farE* expression although this expression is significantly lower than linoleic acid.

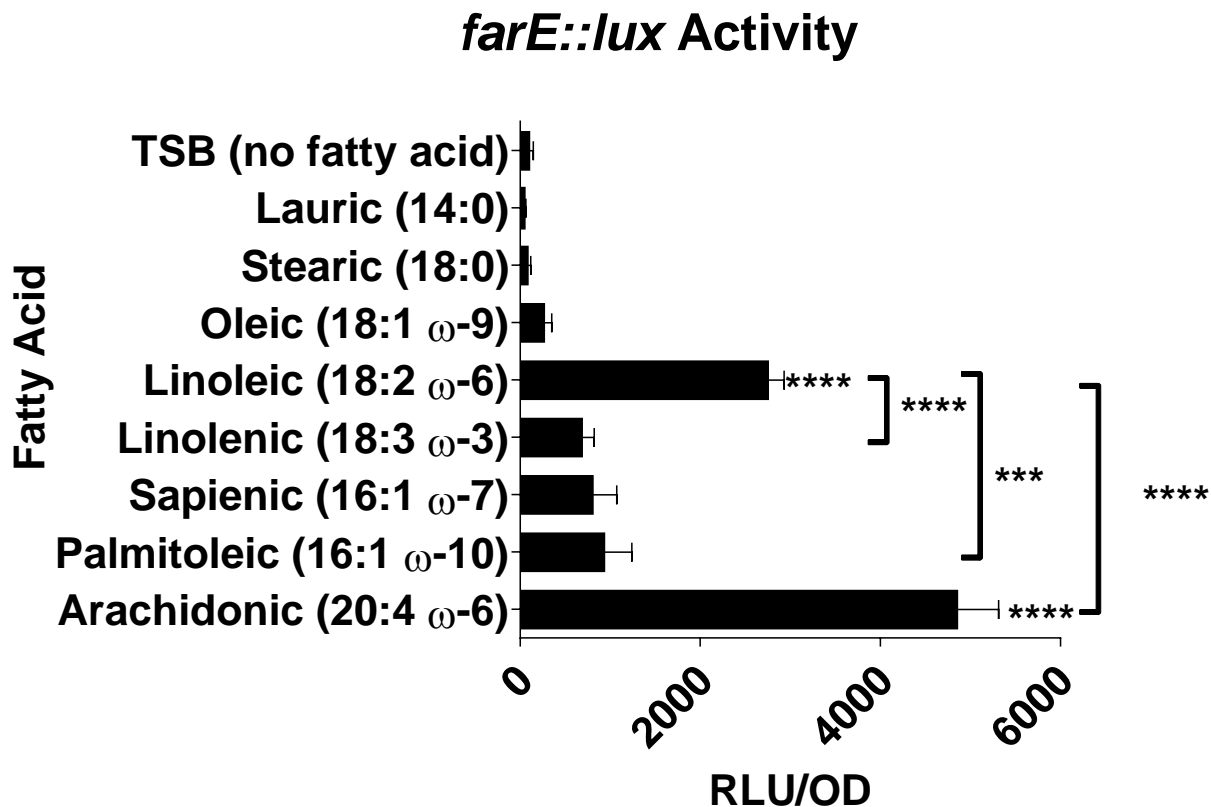


Figure 3.4 Role of different antimicrobial fatty acids on induction of *farE*.

Quantification of pGY*farE::lux* dependent luciferase in USA300 grown to an OD₆₀₀ of ≈ 0.5 in TSB alone or TSB supplemented with 20 μ M of indicated fatty acid. Each value represents the mean of quadruplicate measurements from each of four replicate cultures. P values indicate significant differences in activity compared to TSB alone, or a significant difference between the activity of two different fatty acids. Error bars represented by the Standard Error of the Mean. ***, $p < 0.001$, ****, $p < 0.0001$ determined by one way ANOVA followed by Tukey's multiple comparison test (not all significant differences shown).

3.2.3 *farE* Expression is Not Induced by Non-uFFA Stressors

Upon determining the role of unsaturated free fatty acids on induction of *farE*, we became interested in examining if this response was specific to antimicrobial fatty acids or an aspect of a more generalized stress response. To evaluate this, we compared the levels of *farE* induction by fatty acids to that of non-fatty acid stressors. Several different stressors which inhibit *S. aureus* through different mechanisms were selected, some of which, such as acid, osmotic stress, and oxidative stress, have also been linked to expression of σ^B , an important stress response mediator also shown to be upregulated from exposure to uFFAs (88). These conditions were specifically designed through empirical testing to retard growth to the same extent as 20 μ M linoleic acid. Interestingly, none of these stressors was found to induce *farE* expression to a significant level (Figure 3.5). This suggests that the induction of *farE* is not connected to any generalized stress response but is in response to uFFAs through some unknown sensing mechanism.

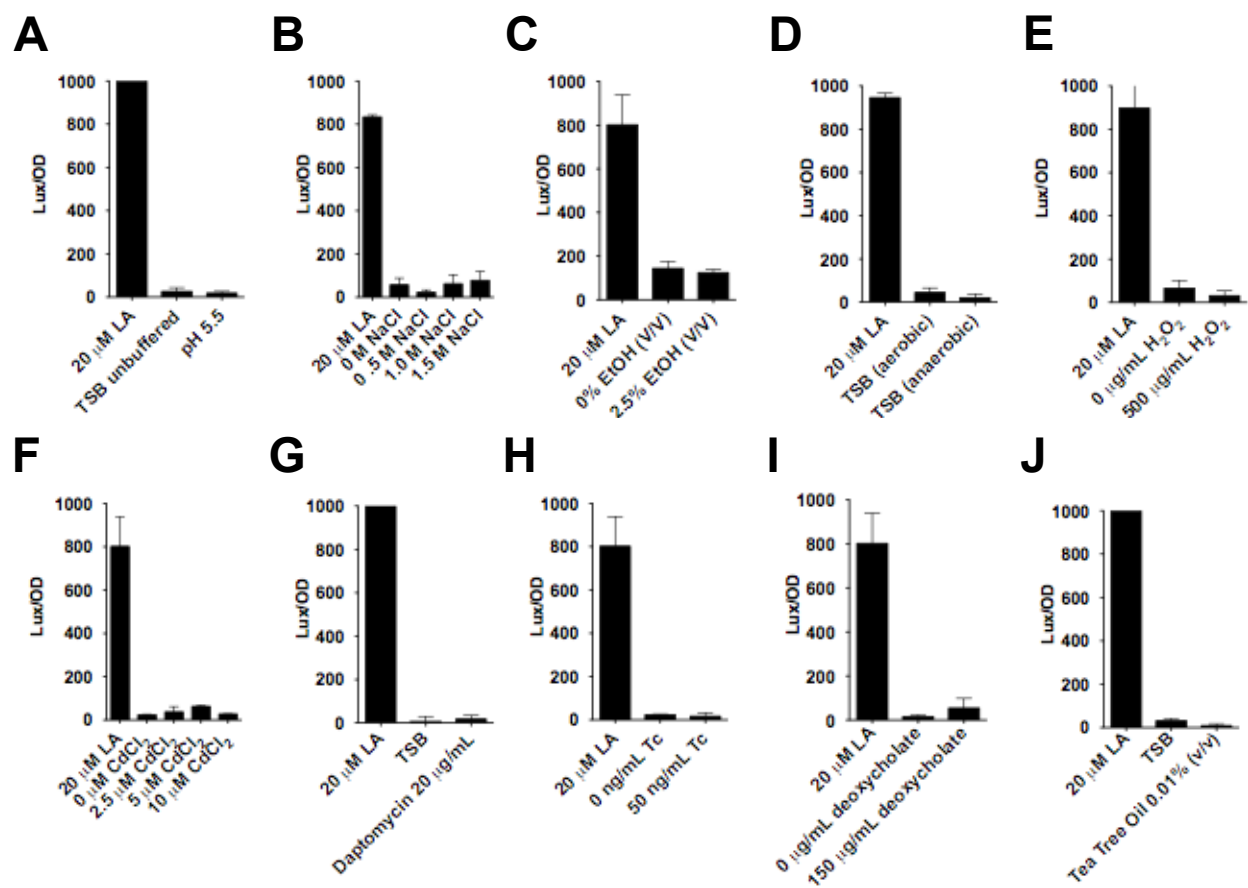


Figure 3.5 Role on Non-Fatty Acid Stressors on *farE* Expression

Quantification of pGY*farE::lux* dependent luciferase in USA300 grown to an OD600 of ≈ 0.5 in TSB alone, TSB supplemented with 20 μ M of linoleic acid, or TSB grown under various conditions to provide comparable stress (noted on graph labels). Each value represents the mean of quadruplicate measurements from each of four replicate cultures. Error bars represented by the Standard Error of the Mean. No significance determined by one-tailed Student's t-test.

3.2.4 Induction of *farE* Promoter is Not Altered in *farE* Deficient USA300

To better understand the mechanism of regulation of *farE*, induction of the *farE* promoter was next evaluated in a strain unable to produce FarE. We were interested in determining whether the inability to produce FarE would alter the level of induction of *farE* compared to a wild-type, which might suggest *farE* is involved in auto-regulation or in a negative feedback loop. To accomplish this, pGY*farE*::*lux* was transformed into USA300*farE*:: Φ NE and monitored for *farE* expression during growth in TSB in the presence and absence of sub-inhibitory levels of linoleic acid (Figure 3.6). Interestingly, it was found that *farE* expression is not significantly different in the *farE* deficient mutant than in wild-type. The expression patterns between these two different strains are very similar to one another, suggesting that the inability to produce FarE does not alter the induction of *farE*.

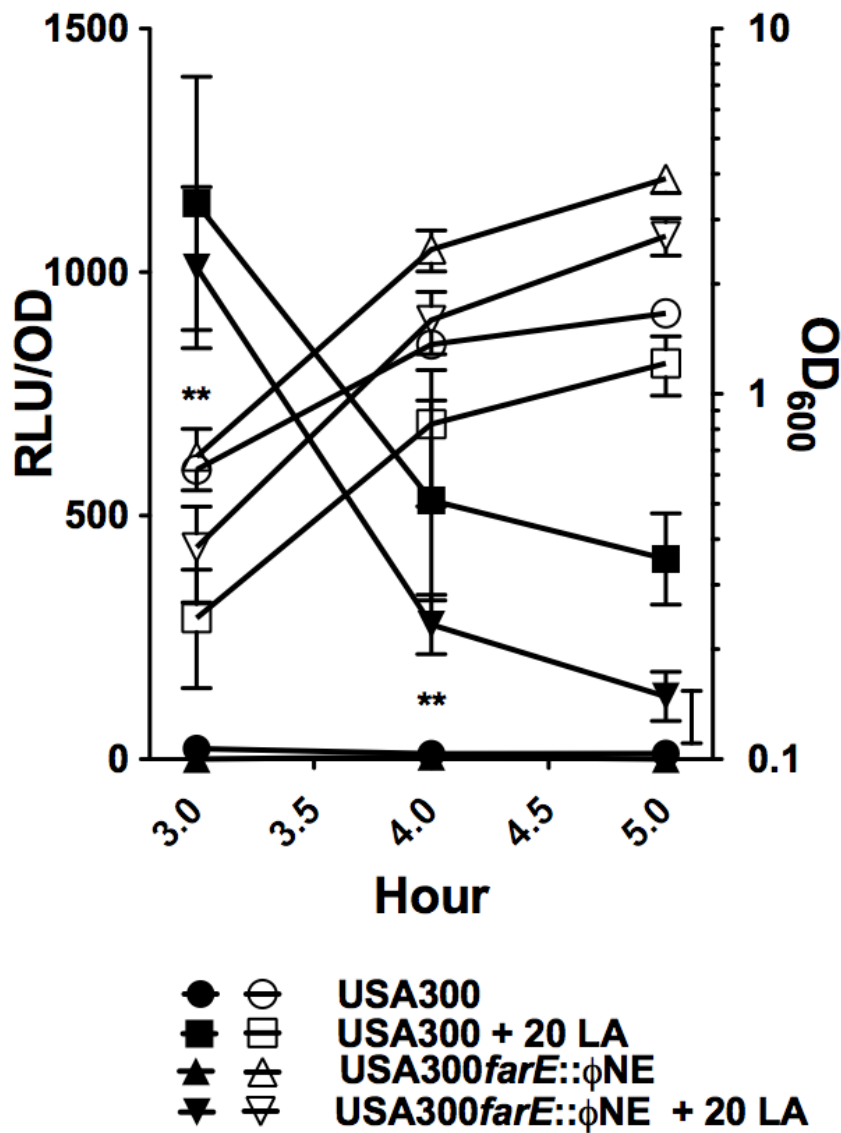


Figure 3.6 *farE* expression unchanged in *farE* deficient USA300

Quantification of pGY*farE*::*lux* -dependent luciferase in USA300 and USA300*farE*::ΦNE grown to an OD₆₀₀ of ≈ 0.5 in TSB alone or TSB supplemented with 20 μ M of indicated fatty acid. Each value represents the mean of quadruplicate measurements from each of four replicate cultures. P values indicate significant differences between USA300*farE*::ΦNE and USA300*farE*::ΦNE + 20 LA. Error bars represented by the Standard Error of the Mean. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ determined by one tailed Student's t-test.

3.3 Evaluation of the Role of *fakA* in uFFA Tolerance and Survival

3.3.1 Confirmation of Lack of α -Hemolysin Production in USA300 Δ *fakA*

Recent literature suggests that *fakA* (also referred to as *vfrB* and *dak2*) has important roles in both *S. aureus* virulence and fatty acid metabolism; mutants deficient in this gene possessed different membrane compositions and formed larger abscesses in *in vivo* invasion models (121, 122). Additionally, a recent study demonstrated that FakA is a component of the fatty acid kinase *S. aureus* utilizes which is necessary to phosphorylate exogenous fatty acids, leading to their incorporation (111). As discussed earlier, *farE* was found to be induced by exposure to fatty acids; however, it is unknown whether the induction requires free fatty acids or if it requires phosphorylated fatty acids that have been incorporated into *S. aureus*. We became interested in how *farE* senses fatty acids, and whether its induction requires phosphorylated fatty acids, and opted to evaluate the role *fakA* might have in tolerance to antimicrobial uFFAs and induction of *farE*.

Previous literature described mutants deficient in *fakA* as causing minimal α -hemolysin production when grown on solid media (122). As a result, the first step was to create USA300 Δ *fakA* and evaluate the α -hemolysis of this strain to confirm the correctness of this mutation. This USA300 Δ *fakA* mutant was then transformed with either pAL*fakA*(+), a complementation plasmid, or pAL*fakA*(-), a control plasmid containing inverted and thus non-functional *fakA* gene. To confirm the previously described α -hemolysis phenotype for the USA300 Δ *fakA* generated by this study, its α -hemolysis activity was also evaluated through 24 hours of growth on a tryptic soy agar plate containing 5% defibrinated rabbit blood (Figure 3.7). USA300 Δ *fakA* + pAL*fakA*(-) demonstrated almost no clearing, with a profile similar to the USA300*hla*:: Φ NE mutant. The USA300*hla*:: Φ NE (NE1354) is unable to produce α -hemolysin and functions as a positive control for loss of hemolysis activity. These findings matched those of the previous studies. Additionally, complementation of *fakA* utilizing the pALC2073 vector, as described in the Materials and Methods, was sufficient to restore the wild-type phenotype. Additionally, to evaluate if *farE* has any impact on α -hemolysin production, a USA300*farE*:: Φ NE mutant was also evaluated. It was

found to demonstrate clearing comparable to the wild-type, suggesting *farE* is unrelated to α -hemolysin production.

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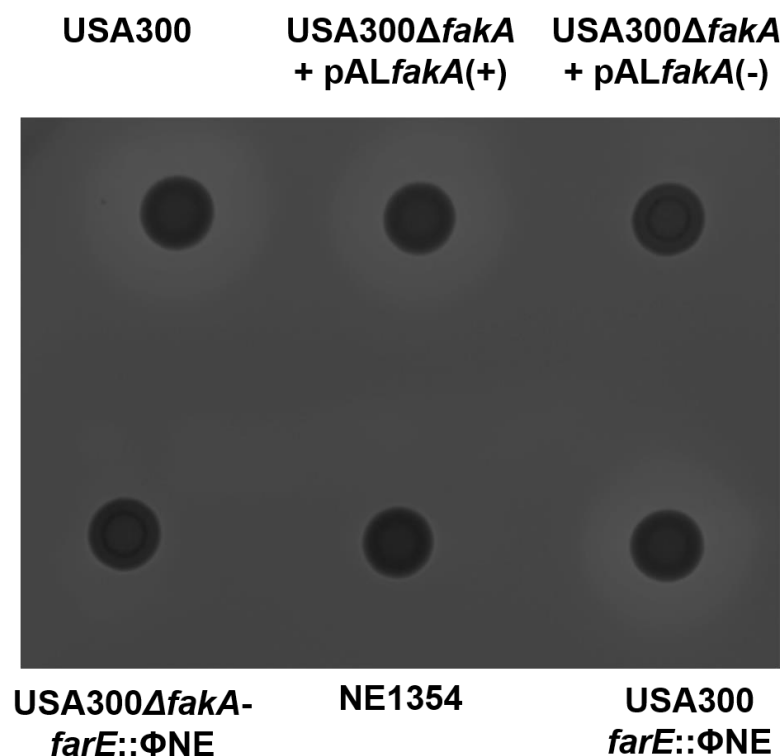


Figure 3.7 Confirmation of reduction of *fakA*-dependent hemolysis activity

Hemolytic activities of: USA300, USA300 Δ *fakA* with either complementation plasmid pAL*fakA*(+) or reversed and non-functional complementation plasmid, pAL*fakA*(-), USA300 Δ *fakA*-*farE*:: Φ NE, NE1354 (*hla*) and USA300*farE*:: Φ NE. Hemolysis is seen in rings of clearance on TSA containing 5% defibrinated rabbit blood, corresponding to destruction of erythrocytes.

3.3.2 *farE* is Expressed Constitutively and is Not Inducible by Linoleic Acid in *fakA* Negative Strains

After confirming the $\Delta fakA$ strain demonstrated the phenotype described by previous literature, we focused next on examining the expression of *farE* in the $\Delta fakA$ deficient mutant. As *fakA* is necessary to phosphorylate exogenous fatty acids, we wanted to evaluate whether this step was important for *farE* expression, and thus evaluate if expression was different from that exhibited by wild-type USA300. To accomplish this, the pGY*farE::lux* plasmid was transformed into USA300 $\Delta fakA$, as described in the materials and methods. USA300 + pGY*farE::lux* and USA300 $\Delta fakA$ + pGY*farE::lux* were subsequently both grown in TSB and sub-inhibitory concentrations of linoleic acid. Strikingly, when USA300 $\Delta fakA$ was grown in TSB under non-inducing conditions, it exhibited significantly elevated *farE::lux* activity in mid-exponential growth compared to wild-type USA300. However, when USA300 $\Delta fakA$ was grown in TSB + 20 μ M LA, there was no additional increase in *farE::lux* activity beyond that of the non-induced growth condition, whereas wild-type USA300 exhibited strong induction of *farE::lux* activity. Cumulatively, this data shows that *farE* is both constitutively expressed in USA300 $\Delta fakA$, and is not further induced by the presence of uFFAs.

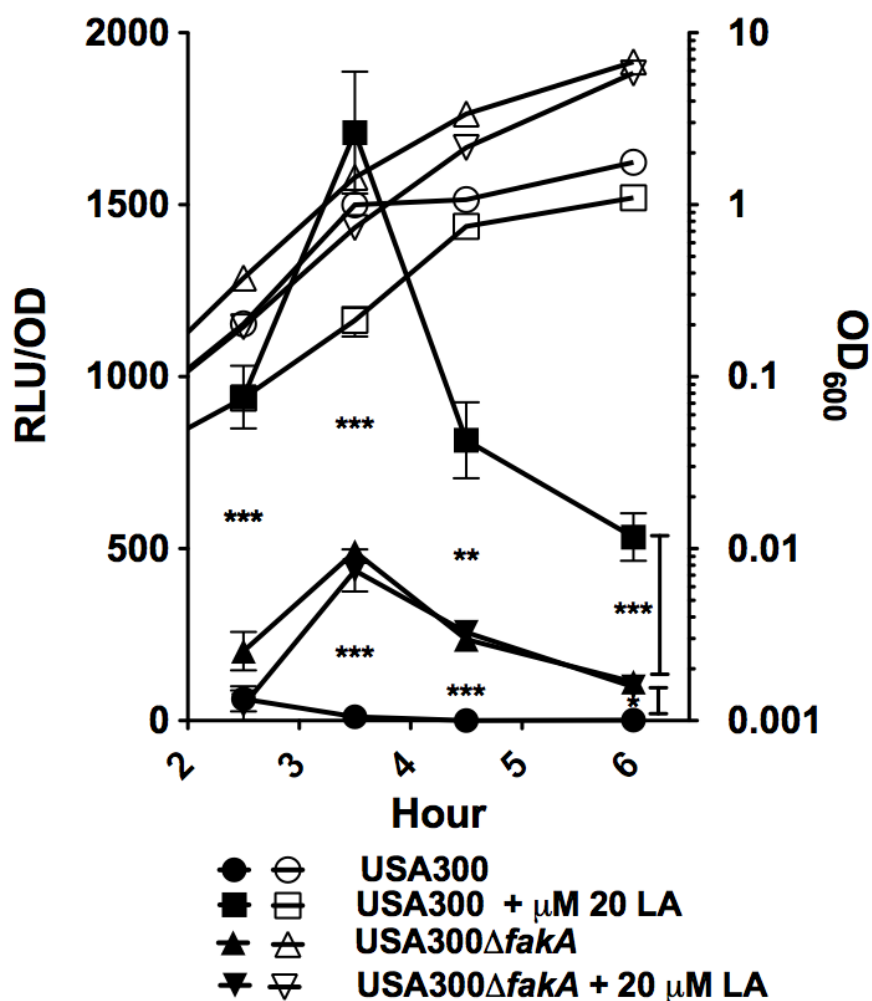


Figure 3.8 *farE* is constitutively expressed in USA300ΔfakA

Growth (OD_{600} ; open symbols) and relative luminescence units normalized by OD (RLU/OD; closed symbols) of USA300 and USA300ΔfakA carrying the pGY*farE::lux* reporter vector are graphed. Strains were grown in TSB alone or in TSB supplemented with 20 μ M linoleic acid. Each value represents the mean and standard deviation of results of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represented by the Standard Error of the Mean. Significance values shown between USA300 and USA300ΔfakA (lower) and USA300 + 20 μ M LA and USA300ΔfakA + 20 μ M LA. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ determined by one tailed Student's t-test.

3.3.3 USA300 Δ *fakA* is Less Susceptible to Killing by Bactericidal Concentrations of Linoleic Acid

After identifying that *farE* is constitutively expressed in USA300 Δ *fakA*, we wanted to determine next if USA300 Δ *fakA* might have greater resistance to killing by antimicrobial fatty acids. To assess the role of *fakA* in resistance to uFFAs, a bactericidal assay was conducted utilizing linoleic acid. Wild-type USA300, USA300 Δ *fakA* + pAL*fakA*(+), and USA300 Δ *fakA* + pAL*fakA*(-) were grown to exponential phase in TSB alone and then inoculated into flasks containing 100 μ M linoleic acid, a bactericidal concentration. Interestingly, the USA300 Δ *fakA* + pAL*fakA*(-) mutants demonstrated significantly higher survival at every time point compared to the wild-type and complement (Figure 3.8). This data shows that mutants which are deficient in *fakA* are less susceptible to killing by bactericidal uFFAs such as linoleic acid. Complementation of *fakA* restored cell killing to levels not significantly different from wild-type USA300.

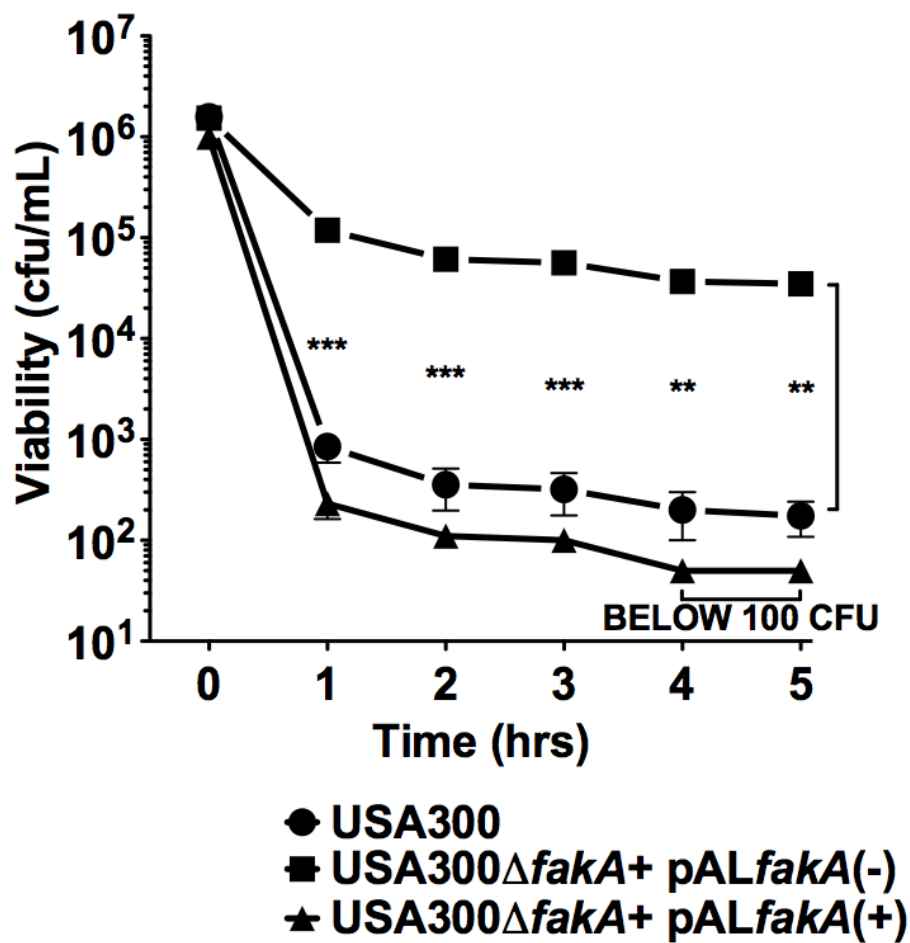


Figure 3.9 USA300ΔfakA more resistant to killing by bactericidal concentrations of linoleic acid

Bactericidal activity of 100 μM linoleic acid measured with USA300, USA300ΔfakA + pALfakA(+) and USA300ΔfakA + pALfakA(-), prepared by growth to mid-exponential phase in TSB. Each data point represents the mean value of quadruplicate cultures. P values for comparison of USA300 and USA300ΔfakA + pALfakA(-) cells are indicated by asterisks (**, $P < 0.01$, ***, $P < 0.001$).

3.3.4 *farE* is an Important Contributor to the Ability of *fakA* Deficient Strains to Resist Killing by Linoleic Acid

Upon determining that *fakA* deficient mutants had significantly higher survival in bactericidal concentrations of uFFA than wild-type *S. aureus*, we became interested in whether the constitutive expression of *farE* was involved in this phenotype. Prior research had demonstrated that when induced in sub-inhibitory concentrations of uFFAs, *farE* deficient strains were significantly more susceptible to killing by bactericidal concentrations of uFFA than wild-type USA300 (108). To evaluate the role of *farE* in the USA300 Δ *fakA* resistance, a strain deficient in *farE* and *fakA* was constructed by transducing the *farE*:: Φ NE mutation into USA300 Δ *fakA*, as described in the Materials and Methods. Bactericidal assays utilizing USA300, USA300 Δ *fakA* and USA300 Δ *fakA*-*farE*:: Φ NE were carried out under the same conditions as previously utilized. Interestingly, USA300 Δ *fakA*-*farE*:: Φ NE had significantly lower viability than USA300 Δ *fakA* (Figure 3.9), while still having significantly higher viability than wild-type USA300. Taken together, this data shows that *farE* is an aspect of the *fakA* resistance to uFFAs, although it is not the only contributor.

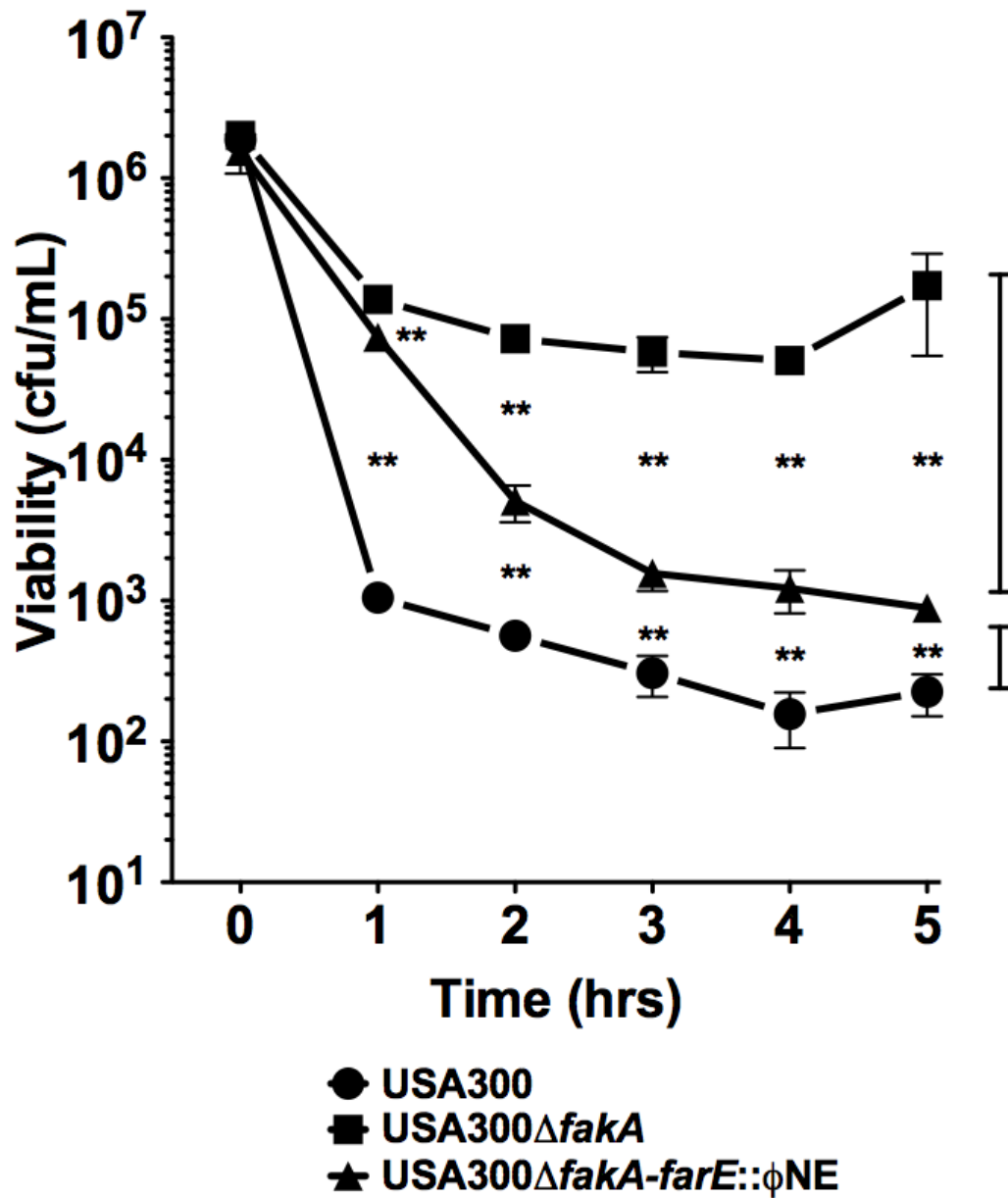


Figure 3.10 *farE* is an important contributor to USA300ΔfakA's improved survival in linoleic acid

Bactericidal activity of 100 μM linoleic acid measured with USA300, USA300ΔfakA and USA300ΔfakA- *farE*::ΦNE, prepared by growth to mid-exponential phase in TSB. Each data point represents the mean value of quadruplicate cultures. *P* values for comparison of induced USA300 and induced FAR7 cells are indicated by asterisks (**, *P* < 0.01, ***, *P* < 0.001).

4 DISCUSSION

The rise in USA300 as an epidemic strain of *S. aureus* is thought to be largely the result of its remarkable ability to overcome innate immune defenses on the skin, notably skin acidity and polyamines (32). Patients deficient in the production of uFFAs are more susceptible to certain *S. aureus* caused skin diseases (42). With this in mind, it seems likely USA300 would have an ability to overcome uFFAs encountered on the skin, as well as in abscesses, through several different strategies.

Previous research by Truong-Bolduc *et al.* identified a member of the Major Facility Superfamily encoded by the gene *tet38* as having the ability to transport palmitoleic acid (103). Palmitoleic acid (16:1 *cis*- Δ^9) is a fatty acid present in most human tissues, and importantly, its isomer sapienic acid (16:1 *cis*- Δ^6) is a major uFFA in human sebum (40). It was found that deletion of this gene in strain MW2 halves the MIC of palmitoleic acid, while over-expression of this gene doubles the MIC of linoleic acid (103). When we generated a deletion mutant of this gene in USA300 during this study however, we did not detect any significant differences in the ability of USA300 to grow and survive in palmitoleic acid. Hypothesizing that expression of *farE* could compensate for the deficiency of *tet38*, we generated a mutant deficient in both of these genes. However, we once again detected no differences in survival to palmitoleic acids, suggesting that the reason we did not detect a similar effect is not as a result of *farE*, but likely *tet38* functions in a strain-specific context. Truong-Bolduc *et al.* evaluated MW2, also known as USA400, and the laboratory strain RN6390, a σ^B deficient mutant derived from *S. aureus* NCTC8325(137). USA300 is a member of a different clonal complex (CC 8) than USA400 (CC1), and despite them together comprising a majority of CA-MRSA infection in the United States, there is a large evolutionary distance between these two strains (138-140). The MFS is an extremely broad group of transporters and many members are capable of efflux of multiple substrates, even substrates which are not structurally related (141).

The protein Tet38 was originally discovered for its role in tetracycline efflux in MW2 (102). Interestingly, *tet38* is located within a cluster of genes annotated for nucleoside and deoxynucleoside catabolic enzymes, flanked on one side by *deoD* and the other by *deoC* and *deoB* (22). These genes act together to degrade ribonucleosides and deoxynucleosides as an additional

source of energy (142). The location of *tet38* within this cluster might suggest the original role of *tet38* could be involved in efflux of compounds arising from nucleotide metabolism. Overall, it appears that different *S. aureus* strains are capable of utilizing this transporter for different substrates. Further research might be necessary to elucidate what the native role of *tet38* truly is.

Additionally, while *farE* promotes resistance to linoleic acid, neither *farE* nor *tet38* were found to have important roles in resistance to palmitoleic acid (108). These findings are consistent with our analysis of the fatty acid induction data, which showed that palmitoleic acid induces significantly lower *farE* expression than linoleic acid or arachidonic acid. This data provides evidence that *farE* is not significantly involved in palmitoleic acid resistance. Interestingly, research in our laboratory has demonstrated that USA300 grown to exponential phase in sub-inhibitory concentrations of palmitoleic acid has a significant resistance to killing by bactericidal concentrations of palmitoleic acid which suggests that there is an inducible response to palmitoleic acid (unpublished data, McGavin). Whether this response is from a mechanism specific to palmitoleic acid or part of a broader uFFA resistance could be a focus of future research.

While our results show that *farE* does not have a role in resistance to palmitoleic acid, despite modest induction by palmitoleic acid and its isomer sapienic acid, one of the most striking findings is the *farE* response to certain uFFAs, notably linoleic acid and arachidonic acids. The large level of induction from linoleic acid supports that linoleic acid is one of the main inducers of *farE* expression. These findings also identify an important and previously unexamined role for arachidonic acid, a major component of cellular membranes but not large component of skin sebum uFFAs. Interestingly, arachidonic acid is present in erythrocyte membranes, comprising about 13% of the fatty acids in membranes, in lymphocytes, which would respond to invasion, it is present in higher concentrations, composing up to 18% of the fatty acids present (56, 143). Thus, arachidonic acid would be present during *S. aureus* invasion and abscesses formation. Subsequent research from our laboratory has confirmed the importance of *farE* in inducible survival in bactericidal concentrations of linoleic and arachidonic acids, as well as confirming linoleic acid as a substrate of FarE (108). Both linoleic acid and arachidonic acid are aspects of the innate antimicrobial uFFAs in nasal secretions (41). Nasal carriage is extremely important for *S. aureus*, and is thought to be ubiquitous in *S. aureus* carriers (10). As a result, it appears *farE* would have an important role in the ability of USA300 to colonize human hosts through tolerance of these fatty acids. Additionally, *farE* might have an important role in USA300 invasion and survival in abscesses.

Fatty acids are found in large concentrations within abscesses, including linoleic acid in murine abscesses (55). Additionally, arachidonic acid and linoleic acids both make up a large component in cellular membranes (56). Through hydrolysis of triglycerides by leukocyte or staphylococcal lipases these fatty acids could be released as uFFAs (55, 57). As a result, linoleic acid and arachidonic acid could comprise a large component of the uFFAs encountered by *S. aureus* within abscesses, making it noteworthy that these are the two strongest inducers of *farE* expression. These suggest *farE* might be a natural evolutionary response specific to the unsaturated fatty acids linoleic and arachidonic, which are encountered during invasion, first within the nares, and then within abscesses. Confirming this through *in vivo* experiments evaluating both ability of *farE* mutants to persist on skin, and establish abscesses, would be a reasonable next step in evaluating this role.

While several different uFFAs were found by this study to induce *farE* expression, strikingly, no non-uFFA stressors were capable of inducing even modest *farE* expression. Other research has identified that uFFA exposure leads to expression of σ^B and the corresponding stress response factors, suggesting that one aspect of the *S. aureus* response to uFFA is a generalized stress response (88). Several of the stressors tested for *farE* induction, such as acid, osmotic stress, and oxidative stress, have also been identified as capable of inducing a σ^B response (85, 87); however, none of these were capable of inducing *farE* expression. Stressors which would damage membranes, the proposed method of anti-microbial action by uFFAs, such as tea tree oil, were similarly unable to induce *farE* expression. These findings suggest that *farE* regulation is distinct from global response and stress regulators such as σ^B and mediated through a fatty acid specific sensing mechanism. Similarly, expression of *farE* was found to be at its highest levels during mid-exponential phase of growth; in *S. aureus*, the global regulators *agr* and *sarA* are most involved in regulation during the stationary phase (76). Taken together, these findings suggest that the expression of *farE* is distinct from global regulators and stress responses and controlled through a uFFA specific mechanism. This study also identified that *farE* expression in USA300 deficient in *farE* was the same as in wild-type USA300 when exposed to uFFAs. It was originally theorized that in the absence of *farE*, fatty acids might accumulate to higher concentrations within the cell and lead to correspondingly higher levels of *farE* expression; interestingly, this was not the case. Our interpretation of this result is that *farE* regulation levels are not based simply on sensing the concentration of free fatty acids, but instead through another mechanism. This is also consistent

with the observation that the same concentrations of linoleic and arachidonic acid were capable of inducing significantly different levels of *farE* expression at the same molar concentrations.

Another finding of this study was the role of *fakA*, which encodes an essential component of the fatty acid kinase, in *farE* induction. *fakA*, along with either *fakB1* or *fakB2*, encode a fatty acid kinase which allows exogenous fatty acids to be incorporated (111). As discussed previously, the metabolic fate of these exogenous fatty acids is thought to be incorporation into the membrane phospholipids. A study by Li *et al* found that *fakA* deficient mutants possessed resistance to dermicidin, an antimicrobial which damages membranes. The resistance to this antimicrobial, which binds to and damages membrane, was thought to be to the result of an altered phospholipid composition. Specifically, the *fakA* mutant exhibited less branched chain fatty acids and more straight chain fatty acids, as well as less diphosphatidylglycerol (cardiolipin), one of the three phospholipid species (121). Li *et. al.* suggested that the change in fatty acid composition might have protected against the membrane damaging effects of dermicidin. A different study by Bose *et. al* identified that *fakA* mutants had greater growth in abscess models and differentially regulated virulence factors, producing less α -hemolysin (122). An abscess contains large amounts of uFFAs; as a consequence, our observations of a *fakA* mutant possessing greater resistance to uFFAs would be consistent with these previous findings (55). As our study identified USA300 Δ *fakA-farE::* Φ NE, *fakA* mutants do possess some *farE*-independent resistance to uFFA; a component of this phenotype could be explained through the alterations in membrane structure. However, our study also found that USA300 Δ *fakA* expresses *farE* at a constitutive level, independent of fatty acid exposure, and that USA300 Δ *fakA* has significantly higher survival in bactericidal fatty acids than USA300 Δ *fakA-farE::* Φ NE. Taken together, these findings support that *farE* is involved in *fakA* tolerance to uFFAs, and that *fakA* has a role in *farE* expression.

The exact role of *fakA* on *farE* expression in *S. aureus* has not been determined but there are some findings in the literature which could help explain this relationship. One simple solution could be FakA acts as a repressor of *farE*, such that when FakA is absent, *farE* is expressed constitutively. However, this idea still raises some questions, as USA300 Δ *fakA* is also unable to upregulate *farE* when exposed to uFFAs and as a result expresses *farE* at levels significantly lower than wild-type USA300, a result which would not be anticipated in FakA served only as a repressor. Instead, we propose a slightly more complex involvement for *fakA* in *farE* regulation. As discovered by Parsons *et al.*, *fakA* is an integral part of the fatty acid kinase, which carries out the first step of

incorporating fatty acids into the cell by phosphorylating them into Acyl-PO₄ (111). Previous work in our lab supports that *farR*, which is divergently transcribed from *farE*, encodes a regulator of *farE* (108). It is possible that this regulation of *farE* requires specific forms of Acyl-PO₄ and not free fatty acids to promote *farE* expression. This would be consistent with the observation that *farE* induction is not promoted by uFFAs in USA300Δ*fakA*. Additionally, regulation of *farE* might occur through sensing of bacterial membrane composition. The bacterium *Pseudomonas aeruginosa* has systems in place which sense membrane composition, using DesT, a transcriptional regulator which senses fatty acid composition in the acyl-coenzyme A pool and regulates expression of a desaturase (144, 145). If *S. aureus* had a similar, unidentified mechanism, it might require *fakA* to incorporate uFFAs into the cell in order for the uFFAs to be sensed. In *S. aureus*, the global regulator system *sae* is of note; recent studies have shown that component SaeS senses currently unidentified human signals, and regulates genes correspondingly (146). Interestingly, SaeS is an intramembrane-sensing histidine kinase, which lacks an extracellular sensory domain (147). As a result, SaeS could act to sense membrane composition and affect regulation through the *sae* system. Strikingly, Parsons *et al.* identified that the genes modified in the *fakA* mutant are also thought to be regulated through this *sae* system (111). If *farE* regulation required sensing of fatty acids which had already been incorporated, it could explain the lack of change in *farE* expression between USA300Δ*fakA* grown in TSB or in sub-inhibitory fatty acids (Figure 4.1). Additionally, as USA300Δ*fakA* was shown to have different membrane phospholipid composition, this could additionally explain the constitutive *farE* expression in TSB alone. Interestingly, in some species, RND family transporters are thought to be utilized to replace fatty acids from membranes as part of maintaining homeostasis (148). Although the mechanism is currently not understood, what is known is that *farE* is constitutively expressed in *fakA* deficient mutants, and this level of expression is not altered by exposure to fatty acids. This contributes in part, although not exclusively, to the greater survival of USA300Δ*fakA* in linoleic acid. Identifying the other aspects of USA300Δ*fakA* survival, and specific regulation of *farE*, remains as a future area of study.

In summary, we have examined the *S. aureus* resistance to unsaturated free fatty acids and the multifactorial response involved in its survival. This study demonstrated that the palmitoleic acid efflux pump encoded by *tet38* in CA-MRSA strain MW2 is not involved in the USA300 tolerance of palmitoleic or linoleic acids. Additionally, it identified that *farE*, which encodes a fatty acid

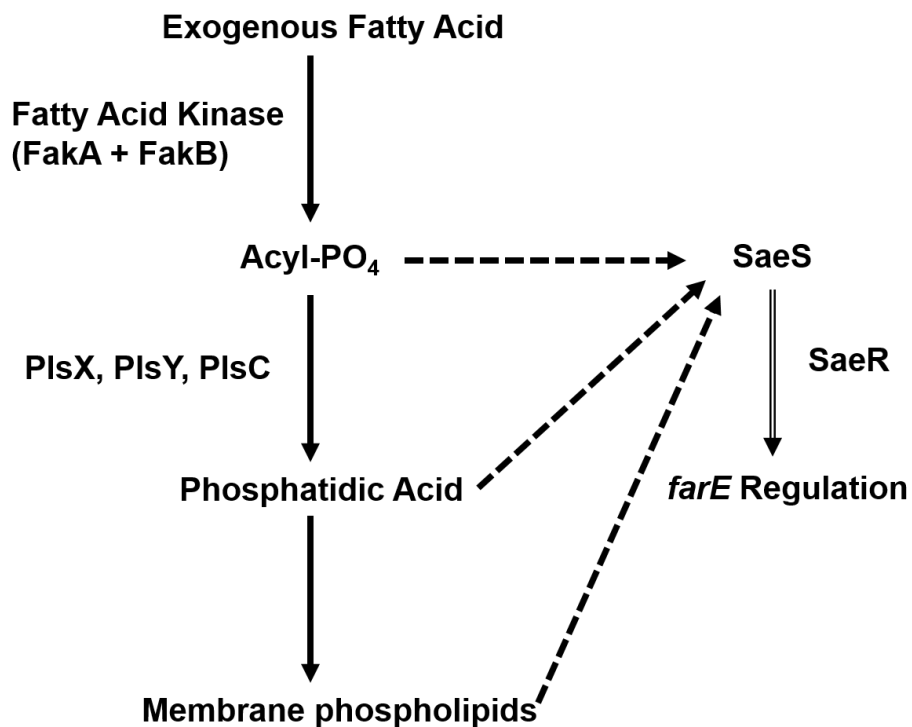


Figure 4.1 Proposed FakA-dependent Sensing of Exogenous Fatty Acids

In this proposed system, exogenous fatty acids can be sensed by SaeS subsequent to their phosphorylation by Fak. It is unclear at which point SaeS evaluates composition of fatty acids, although it is noteworthy in *P. aeruginosa* DesT senses Acyl-PO₄ composition. SaeS then acts through effector SaeR to effect *farE* regulation, possibly involving *farR*, as well as other SaeR regulated genes. Figure adapted from Parsons *et al.* (111).

efflux pump, is induced specifically by uFFAs, notably linoleic and arachidonic acid, two fatty acids found in nasal secretions and abscesses, but not by other stressors which induce a general stress response. *farE* is constitutively expressed in mutants deficient in *fakA*, a gene which encodes a kinase to incorporate exogenous fatty acids. This USA300 Δ *fakA* mutant was also maintained significantly higher viability in bactericidal concentrations of uFFAs than wild-type USA300. While the specific mechanisms underlying *farE* expression remain undefined, this study has identified the specificity of the *farE* response to uFFAs which *S. aureus* would encounter in colonization and invasion.

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